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# Characterization of the Tight Junction Protein ZO-3: Binding Interactions, Role in Junctional Complex Assembly, and Effects on Actin Cytoskeleton Dynamics

by

Erika S. Wittchen

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Cell Biology

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# ABSTRACT

The tight junction is a regulated semi-permeable barrier between adjacent epithelial or endothelial cells which acts to separate the apical and basolateral fluid compartments on opposite sides of a cell monolayer. The goal of this thesis project was to gather information about the binding interactions between various tight junction proteins, and more specifically, to begin to elucidate the function of the tight junction protein ZO-3. In Chapter 2 I show that ZO-2, ZO-3, and occludin interact directly with actin filaments, but do not act as crosslinkers. I also demonstrate that two separate ZO-1/ZO-2 and ZO-1/ZO-3 complexes exist *in vivo*, not a three-part ZO-1/ZO-2/ZO-3 complex as previously thought. Chapter 3 explores further the function of the tight junction protein ZO-3. Results indicate that while the targeting information required for correct localization of ZO-3 to the membrane is contained in the amino-terminal half of the protein (NZO-3), expression of this half of the molecule in MDCK cells perturbs junctional complex assembly. Transepithelial resistance (TER) recovery and tight junction protein recruitment to the membrane is delayed in a calcium switch experiment; additionally, F-actin and the adherens junction proteins E-cadherin and beta-catenin also have delayed recruitment. NZO-3 expression has no effect on junctional protein expression levels with the exception of beta-catenin, which exhibits an increase in its soluble pool at early time points of junctional complex assembly. F-actin and ZO-1 bind preferentially to NZO-3, while occludin and cingulin bind equally to both halves of the protein. In Chapter 4 I show that NZO-3 expression has other effects which affect actin cytoskeleton dynamics within the cell; NZO-3-expressing cells have fewer stress fibers and fewer and smaller focal adhesions than untransfected MDCK cells. This phenotype

correlates with enhanced cell migration and decreased activity of RhoA in NZO-3/MDCK cells. Finally, I show that the COOH-terminal half of ZO-3 binds to AF-6 and p120 catenin, which has implications regarding the potential mechanism for the observed decrease in RhoA activity in NZO-3-expressing cells. In conclusion, the results presented in this thesis provide new information about junctional complex assembly and function.

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*"A successful person is a dreamer whom someone believed in."* -- Anonymous

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## ABBREVIATIONS

| | |
|---|---|
| cD | cytochalasin D |
| COOH- | carboxy |
| EM | electron microscopy |
| GAP | GTPase activating protein |
| GEF | guanine nucleotide exchange factor |
| GDP | guanine nucleotide diphosphate |
| GTP | guanine nucleotide triphosphate |
| GSB | gel sample buffer |
| GST | glutathione *S*-transferase |
| GUK | guanylate kinase |
| HRP | horseradish peroxidase |
| JAM | junctional adhesion molecule |
| MAGUK | membrane-associated guanylate kinase homologue |
| MDCK | Madin-Darby canine kidney |
| N- | amino |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PDZ | PSD95/dlg/ZO-1 |
| PI's | protease inhibitors |
| RBD | Rho binding domain |
| RT | room temperature |
| SH3 | Src homology 3 |
| TBS | Tris buffered saline |
| TER | transepithelial electrical resistance |
| VSVG | vesicular stomatitis virus glycoprotein |
| ZO | zonula occludens |

# CHAPTER 1

## Introduction

## 1.1. The Tight Junction: Physiological Function

Epithelial and endothelial cell monolayers act to separate two biologically distinct compartments such as in the gastrointestinal tract, where the intestinal epithelium is the primary barrier separating the underlying blood capillaries from the lumen of the intestine; or in the case of the blood-brain barrier, where capillary endothelial cells limit the passage of blood-borne agents into the cerebral space. In the case of the small intestine, absorption of nutrients is regulated via two distinct routes: the transcellular pathway, where substances are vectorially transported through the cytoplasm via specific transporter proteins (transcytosis); and the paracellular pathway, where molecules selectively pass through the spaces between adjoining cells (paracytosis). The tight junction is the structural element of epithelial and endothelial cells that forms a physical barrier to the free diffusion of molecules through the paracellular space between cells. The tight junction also acts in the maintenance of apical versus basolateral plasma membrane compositional asymmetry by restricting the movement of lipids and integral membrane proteins within the plane of the membrane. Because of this membrane polarity, the electro-osmotic gradients required for intestinal absorption can be created, and then are maintained because the tight junction barrier prevents the passive backflow of these transported solutes, ions and water (Mitic *et al.*, 2000) through the paracellular space. The tight junction is not a static barrier, but demonstrates plasticity and selectively regulated permeability. Additionally, depending on the tissue, tight junctions can have varying degrees of permeability: from the extremely leaky tight junctions of the kidney proximal tubule (Kottra and Fromter, 1983), to the relatively impermeable tight junctions of blood-brain barrier endothelial cells.

## 1.2. Morphology of the Tight Junction

The tight junction was originally identified as a morphological entity by Farquhar and Palade in 1963 in their seminal paper on junctional complexes in epithelial cells (Farquhar and Palade, 1963). Then called the "zonula occludens," the tight junction was described as a series of apparent membrane fusion points between adjacent epithelial cells, located at the apical-most region of the lateral cell surfaces. An electron-dense plaque of material also characterizes the cytoplasmic surface of the tight junction, and it is now known to be composed of cytoplasmic tight junction-associated proteins and F-actin (Farquhar and Palade, 1963; Hirokawa and Tilney, 1982; Madara, 1987).

Another method of visualizing the morphology of the tight junction is by freeze-fracture electron microscopy. This technique creates a fracture plane that runs between the inner and outer leaflet of the plasma membrane, and it is particularly useful for visualizing structures that lie within the plane of the membrane such as integral membrane proteins. By freeze-fracture the tight junction appears as a set of branching strands and particles that encircle the cell near the apical surface. It is now widely accepted that the tight junction strands visualized by freeze-fracture EM are composed of integral membrane proteins such as occludin and the claudins (Furuse *et al.*, 1996; Furuse *et al.*, 1998a).

## 1.3. Molecular Composition of the Tight Junction

This section describes the protein constituents of the tight junction, beginning with the known integral membrane proteins, followed by the peripheral membrane proteins that localize to the cytoplasmic plaque of the tight junction, and finishing with a section about tight junction-associated F-actin. For reference, Table 1.1 is a complete

listing in chronological order of the proteins that have been localized to tight junctions to date.

*1.3.1. Integral Membrane Proteins*

The identification of transmembrane components of the tight junction is important since they extend into the paracellular space and therefore are likely to be important for junctional barrier function. Occludin was the first tight junction integral membrane protein identified (Furuse *et al.*, 1993). Occludin is a four-pass transmembrane protein with exclusive localization to the tight junction, and occludin antibodies specifically label the tight junction strands observed in freeze-fracture (Furuse *et al.*,1996). Occludin has been directly implicated in forming the actual paracellular barrier in epithelial cells via homophilic interactions between occludin molecules on adjacent cells (Furuse *et al.*, 1998b), and when expressed in fibroblasts, occludin confers cell-cell adhesion (Van Itallie *et al.*, 1997). However, it became apparent that occludin was not the only transmembrane protein of the tight junction, since occludin-deficient embryonic stem cells could differentiate into polarized epithelial cells containing functional tight junctions (Saitou *et al.*, 1998). In addition, when occludin knockout mice were generated, the mice were viable, had morphologically normal tight junctions, and the barrier function of the intestinal epithelium was unaffected (Saitou *et al.*, 2000). These observations fueled the search for additional transmembrane tight junction proteins.

Claudin-1 and claudin–2 were the next tight junction integral membrane proteins to be identified (Furuse *et al.*, 1998a). Like occludin, they contain four transmembrane domains, but bear no sequence similarity to occludin. When claudins are introduced into fibroblasts (which do not form tight junctions), tight junction strands are reconstituted

Table 1.1. Tight Junction-Associated Proteins

| Tight Junction Proteins | Localization | Reference |
|---|---|---|
| **Integral Membrane Proteins:** | | |
| Occludin | immunoEM | Furuse et al., 1993 |
| Claudin family | immunoEM | Furuse et al., 1998 |
| JAM (junctional adhesion molecule) | immunoEM | Martin-Padura et al., 1998 |
| CAR (coxsackie/adenovirus receptor) | immunoEM | Cohen et al., 2002 |
| ESAM (endothelial specific) | immunoEM | Nasdala et al., 2002 |
| **Peripheral Membrane Proteins:** | | |
| ZO-1 | immunoEM | Stevenson et al., 1986 |
| Cingulin | immunoEM | Citi et al., 1988 |
| Spectrin | (see note 1) | Itoh et al., 1991 |
| 7H6/barmotin | immunoEM | Zhong et al., 1993 |
| ZO-2 | immunoEM | Jesaitis & Goodenough, 1994 |
| Rab3b | immunoEM | Weber et al., 1994 |
| Symplekin | immunoEM | Keon et al., 1996 |
| AF-6 (afadin) | (see note 2) | Yamamoto et al., 1997 |
| ZO-3 | immunoEM | Haskins et al, 1998 |
| ASIP (Par-3) | immunoEM | Izumi et al., 1998 |
| atypical PKC | immunoEM | Izumi et al., 1998 |
| Sec6/8 | (see note 1) | Grindstaff et al., 1998 |
| MAGI-1/BAP-1 | immunoEM | Ide et al., 1999 |
| VAP-33 | (see note 1) | Lapierre et al., 1999 |
| Caveolin-1 | immunoEM | Nusrat et al., 2000 |
| huASH-1 | (see note 1) | Nakamura et al., 2000 |
| Par-6 | (see note 1) | Johansson et al., 2000 |
| Pilt | immunoEM | Kawabe et al., 2001 |
| MUPP-1 | immunoEM | Hamazaki et al., 2002 |
| JEAP | immunoEM | Nishimura et al., 2002 |
| F-actin | immunoEM | Madara, 1987 |

note 1: ImmunoEM data localizing these proteins conclusively to the tight junction is still lacking.

note 2: AF-6 has also been localized by immunoEM to the adherens junction (Mandai *et al.*, 1997).

The references refer to the paper that first reported the localization of the protein to the tight junction.

(Furuse *et al.*, 1998b), and cell-cell interactions develop via homophilic or heterophilic interactions between claudin proteins on adjacent epithelial cells (Furuse *et al.*, 1999). To date, there are at least 18 members of the claudin multigene family (Morita *et al.*, 1999a) of proteins including paracellin-1 (claudin-16), which is involved in selective renal $Mg^{2+}$ resorption (Simon *et al.*, 1999), OSP/claudin-11, present in oligodendrocytes and Sertoli cells (Morita *et al.*, 1999b), endothelial-specific claudin (claudin-5) (Morita *et al.*, 1999c), and receptors for the *Clostridium perfringens* enterotoxin (both claudin-3 and claudin-4) (Sonoda *et al.*, 1999).

A third tight junction-associated integral membrane protein, called junctional adhesion molecule (JAM) (Martin-Padura *et al.*, 1998), is a member of the immunoglobulin superfamily of proteins. Not only does JAM promote adhesive activity via homophilic binding interactions when transfected into non-adherent cells, but treatment with anti-JAM antibodies inhibits monocyte transmigration through the endothelial cell monolayer (Martin-Padura *et al.*, 1998). Thus JAM appears to play a role in transmigration/extravisation of monocytes across endothelial cell monolayers at the level of the tight junction; a similar role is assigned to the cell adhesion molecule PECAM (Muller *et al.*, 1993), which is located more basally along the lateral cell surface, and the two proteins likely act in a coordinated manner to facilitate monocyte infiltration along the entire length of the intercellular cleft. Further support for this comes from the fact that JAM binds to the leukocyte integrin, LFA-1 (lymphocyte function-associated antigen 1) (Ostermann *et al.*, 2002). Two other JAM isoforms have now been isolated: JAM-2/VE-JAM named for its specificity for vascular endothelium (Palmeri *et al.*, 2000;

Aurrand-Lions *et al.*, 2001a), and JAM-3 which is highly concentrated in lymphatic endothelial cells and HEVs (high endothelial venules) (Aurrand-Lions *et al.*, 2001b).

Most recently, the coxsackie and adenovirus receptor (CAR) has been identified as a component of the tight junction by immunoelectron microscopic localization, and co-immunoprecipitation with ZO-1 (Cohen *et al.*, 2001). Expression of CAR promotes cell-cell adhesion, as is the case with the junction transmembrane proteins claudin and occludin (Kubota *et al.*, 1999; Van Itallie *et al.*, 1997). CAR expression also increases the electrical barrier (TER) as do JAM and occludin (Aurrand-Lions *et al.*, 2001a; McCarthy *et al.*, 1996). Furthermore, viral infection is dependent on CAR localization; when CAR is sequestered at the tight junction, viruses cannot gain entry. However, when tight junctions are disrupted by treatment with EDTA, CAR becomes accessible for virus docking and infection (Cohen *et al.*, 2001). The fact that JAM has also been identified as a receptor for reovirus (Barton *et al.*, 2001) suggests that at least partial loss of epithelial integrity, or localized tight junction disruption may be a general prerequisite for viral infection.

*1.3.2. Peripheral Membrane Proteins: The MAGUK Family*

The first protein localized to the tight junction was the cytoplasmic plaque protein, ZO-1 (zonula occludens-1) (Stevenson *et al.*, 1986; Anderson *et al.*, 1988), and it was this discovery that initiated the search for all other tight junction-associated proteins. Together with ZO-1, two other proteins that localize to the tight junction, ZO-2 (Jesaitis *et al.*, 1994; Beatch *et al.*, 1996) and ZO-3 (Haskins *et al.*, 1998), have been identified that are members of the MAGUK family of proteins (Membrane-Associated Guanylate Kinase) (Anderson, 1996). The founding member of the MAGUK family is

the *Drosophila* discs large-1 (*dlg*) protein, which localizes to the septate junction (invertebrate homologue of the tight junction), and is a tumor suppressor that when mutated causes neoplastic overgrowth of the larval imaginal discs (Woods and Bryant, 1991).

ZO-1, ZO-2, and ZO-3 contain a conserved series of modular protein domains (Figure 1.1). They each possess three amino-terminal PSD95/dlg/ZO-1 (PDZ) domains (Cho *et al.*, 1992; Woods and Bryant, 1991; Willott *et al.*, 1993), followed by a Src homology 3 (SH3) domain (Mayer *et al.*, 1993) and a region homologous to yeast guanylate kinase (Stehle and Schulz, 1990), the GUK domain. ZO-1, ZO-2, and ZO-3 each also have an acidic domain, a basic arginine-rich region (~17% in ZO-3) between PDZ1 and PDZ2, and a proline-rich region (~13-14%) that is not seen in other MAGUK family members (Dimitratos *et al.*, 1999). In ZO-1 and ZO-2, the proline-rich region is in the carboxy-terminal portion of the protein; in ZO-3 it is located in the amino-terminal half of the protein between PDZ2 and PDZ3.

*1.3.3. MAGUK Protein Family Domain Function*

The domains of MAGUK proteins comprise well-characterized protein interaction motifs, and the modular nature of MAGUK protein domain organization makes these proteins well suited to act as molecular scaffolds, mediating the assembly of macromolecular complexes via protein-protein interactions. For example, PDZ domains can either interact with the specific amino acid sequences at the COOH-terminus of integral membrane proteins, or can dimerize with other PDZ domains (Sheng and Sala, 2001). Binding of PDZ-containing proteins to membrane receptors or ion channels can

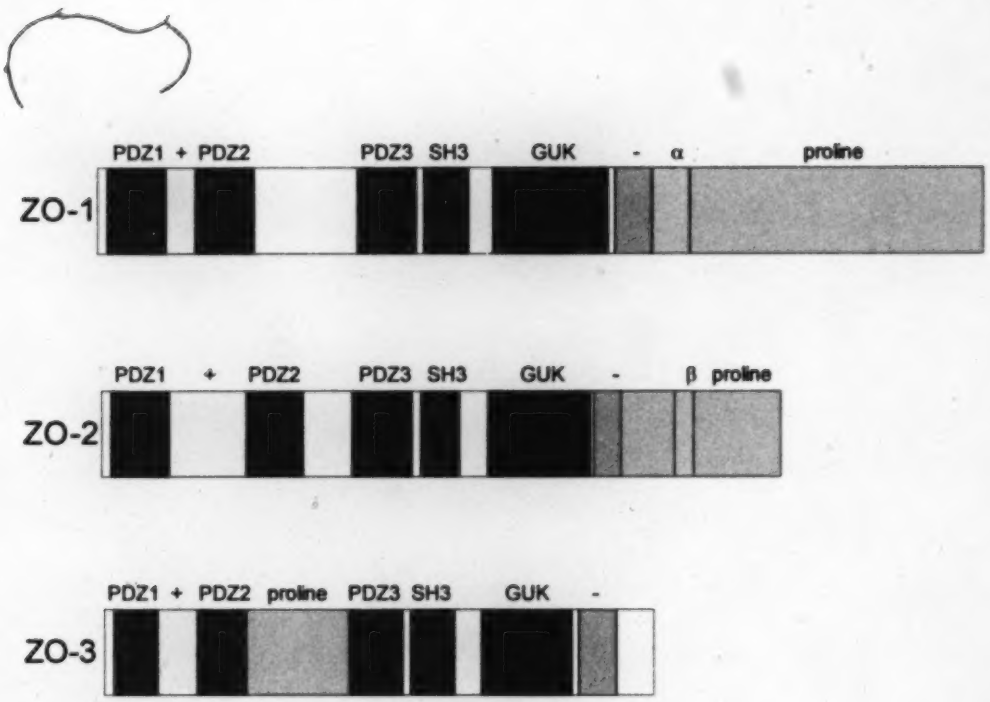


**Figure 1.1. Domain arrangement of three MAGUK proteins: ZO-1, ZO-2, and ZO-3.** ZO-1, ZO-2, and ZO-3 each have three PDZ domains, an arginine-rich (basic) domain (+), and SH3 domain, a GUK domain, an acidic region (-), and a proline-rich region. ZO-1 and ZO-2 each have an alternate splice site (α and β). The proline-rich region in ZO-3 is located between PDZ2 and PDZ3, not at the extreme COOH-terminus as in ZO-1 and ZO-2.

induce clustering of these membrane proteins, thus aiding in the generation of signal transduction cascades. An example of this is the *Drosophila* MAGUK protein Dlg-1 binding to $K^+$ channel proteins (Tejedor *et al.*, 1997).

SH3 domains also participate in protein-protein interactions by binding to the core motif PXXP, with specificity conferred by the amino acids flanking these four (Ren *et al.*, 1993). SH3 domains can participate in both intermolecular and intramolecular binding interactions. The most cited example of an SH3 domain intermolecular interaction occurs between the adaptor molecule Grb2 (SH3 domain-containing) and the guanine nucleotide exchange factor Sos during membrane recruitment in the Ras activation pathway (Rozakis-Adcock *et al.*, 1993). In some instances, SH3 domains can also interact with binding sites on the same protein. These intramolecular interactions involving SH3 domains often act as molecular switches, as in the case of Src. Here the SH3 domain binds to a downstream linker region, keeping the kinase in an inactive conformation until activating phosphorylation events occur to release the SH3 binding interaction (Mayer *et al.*, 2001). Recently it has been shown that this type of regulatory intramolecular interaction occurs in the MAGUK protein PSD-95, involving the SH3 domain and GUK domain (Tavares *et al.*, 2001). The authors speculate this is a conserved feature among all MAGUK proteins that could be important in regulating binding interactions with other proteins.

The GUK domain is named for its homology to yeast guanylate kinase, which catalyzes the conversion of GMP to GDP using ATP as the phosphate donor. However, most members of the MAGUK family including ZO-1, -2, and -3 have a 3 amino acid deletion in the ATP binding site which renders the enzyme inactive (Koonin *et al.*, 1992).

The absence of catalytic activity not withstanding, the GUK domain has also been identified as an important domain for protein-protein interactions (Satoh *et al.*, 1997; Takeuchi *et al.*, 1997).

*1.3.4. Non-MAGUK Peripheral Membrane Proteins*

Other peripheral membrane proteins definitively localized to the cytoplasmic surface of the tight junction by immunoelectron microscopy include cingulin (Citi *et al.*, 1988), symplekin (Keon *et al.*, 1996), barmotin/7H6 (Zhong *et al.*, 1993; Muto *et al.*, 2000), MAGI-1/BAP-1: (membrane-associated guanylate kinase with an inverted arrangement of protein-protein interaction domains/BAI-associated protein), (Dobrosotskaya *et al.*, 1997; Ide et al, 1999), pilt (protein incorporated later into tight junctions) (Kawabe *et al.*, 2001), and caveolin-1 (Nusrat *et al.*, 2000). While caveolin-1 is not exclusively a tight junction protein, it co-localizes with occludin by immunoelectron microscopy, and it is also known to organize specific lipid microdomains (lipid rafts) that are found at tight junctions (Nusrat *et al.*, 2000). Other proteins that have not been definitively localized to the tight junction by immunoelectron microscopy, but may still be tight junction-associated proteins include MUPP-1 (Hamazaki *et al.*, 2002), huASH-1, a putative transcription factor with dual nuclear/cell membrane localization (Nakamura *et al.*, 2000), spectrin (Itoh *et al.*, 1991), and the Ras target AF-6 (Yamamoto *et al.*, 1997). The exact localization of AF-6 remains under debate, with some investigators reporting that it is found at the adherens junction in rat small intestine (Mandai *et al.*, 1997), and others localizing it to the tight junction in MDCK cells (Yamamoto *et al.*, 1997).

A number of proteins that associate with the tight junction also have roles in membrane trafficking events such as Rab3B (Weber *et al.*, 1994), and VAP-33 (Lapierre *et al.*, 1999). That these proteins participate in regulation of membrane traffic in the biosynthetic/endocytic pathways, raises the possibility that their recruitment to the tight junction may specify vesicle targeting and docking sites that are important for tight junction assembly protein recruitment. Sorting of apical and basolateral proteins is also essential for development of epithelial cell polarity. It has been suggested that the Sec6/8 complex specifies delivery of vesicles containing basolateral proteins, and that this requires cell-cell adhesion and recruitment of this complex to the lateral membrane, possibly to the tight junction (Grindstaff *et al.*, 1998). Another polarity-related protein, PAR-6, can form a multiprotein complex with ASIP(PAR-3) and active Cdc42 or Rac, and it associates with the tight junction (Johansson *et al.*, 2000). It is thought that the PAR-6/ASIP(PAR-3) complex is recruited and tethered to the tight junction via a direct interaction with the tight junction protein JAM (Ebnet *et al.*, 2001; Itoh *et al.*, 2001). Furthermore, ASIP(PAR-3) interacts with, and recruits atypical PKCζ and atypical PKCλ to the tight junction ( Izumi *et al.*, 1998).

*1.3.5. F-actin*

Although not exclusively localized to the tight junction, it has long been known that actin filaments are associated with the cytoplasmic surface of the tight junction (Hirokawa and Tilney, 1982). Electron microscopic studies revealed that actin filaments integrate into the tight junction by inserting into the electron dense cytoplasmic plaque specifically at the areas subadjacent to the sites of membrane fusion points (Madara, 1987). Further evidence that the actin cytoskeleton might be the anatomical basis for

controlling tight junction permeability has come from many different studies using F-actin-disrupting drugs such as cytochalasin D. As an example, treatment with cytochalasin D decreases the number and complexity of tight junction freeze-fracture strands in intestinal epithelium (Madara *et al.*, 1986). MDCK cells treated with cytochalasin D demonstrated decreased TER, concomitant with disruption of the apical perijunctional ring of F-actin into large punctate aggregates, which also contain redistributed ZO-1 (Stevenson and Begg, 1994.)

How does the association of the actin cytoskeleton with the tight junction translate into a mechanism for regulating paracellular permeability? Solute-induced opening of tight junctions causes large dilations between the freeze-fracture tight junction strands, and under these conditions investigators have observed condensed microfilaments in the zone of the perijunctional actomyosin ring, which indicates tension is being generated (Madara and Pappenheimer, 1987). This contraction of the actin ring may then cause structural alterations within the tight junction, and increase paracellular permeability. Further support for this model comes from studies where expression of constitutively active myosin light chain kinase causes contraction of cortical actin along with decreased TER and increased paracellular flux (Hecht *et al.*, 1996).

### 1.4. Binding Interactions

A schematic representation of the key binding interactions among both tight junction and adherens junction-associated proteins relevant to this thesis is shown in Figure 1.2. The identification of some of these interactions will be discussed in later chapters.

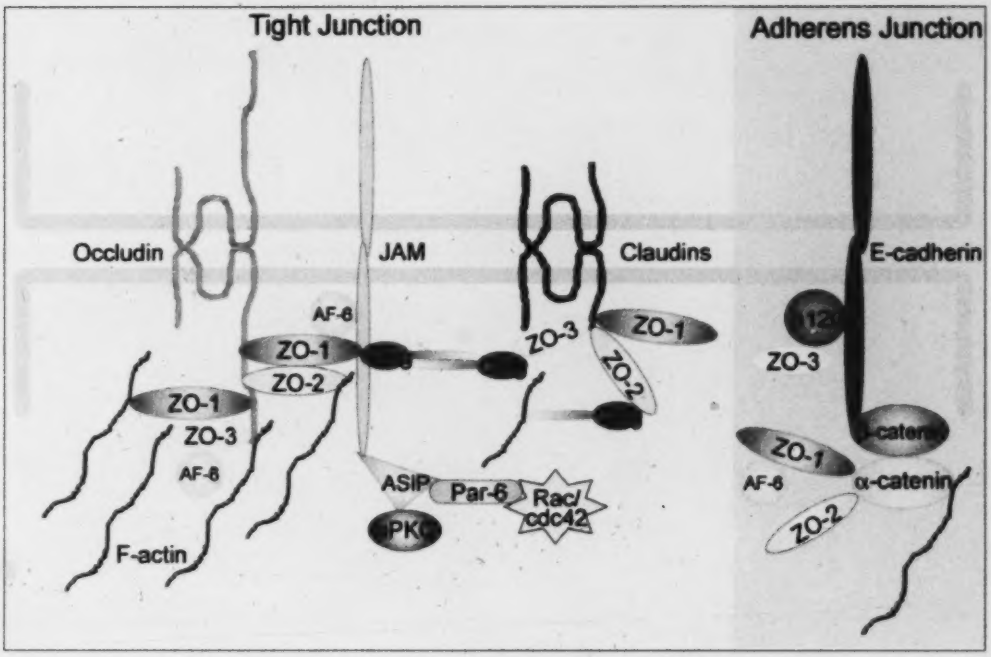


**Figure 1.2. Schematic representation of some key binding interactions among tight junction and adherens junction proteins.** This schematic highlights the degree of complexity of protein-protein interactions found at both junctions, but whether these interactions all occur simultaneously in a physiological context is not known. Note that while the tight junction and adherens junction are distinct structures, there is a cross-interaction between certain junctional proteins (e.g., ZO-1 and ZO-2 binding to α-catenin).

*1.4.1. ZO protein binding interactions*

The first information to be gained about binding interactions of tight junction proteins was that ZO-1, ZO-2 and ZO-3 can be coimmunoprecipitated from MDCK cells (Balda *et al.*, 1993; Jesaitis *et al.*, 1994; Haskins *et al.*, 1998). This suggested the presence of a protein complex, but the exact configuration of that complex was unknown at that time. Based on immunoprecipitation of protein constructs from transfected cells, some investigators suggested that ZO-1 and ZO-2 interact with each other (Fanning *et al.*, 1998; Itoh *et al.*, 1999a). However, these binding studies were performed with whole cell lysates, and the possibility that the interaction is indirect and mediated by other proteins cannot be excluded. Our laboratory has shown via *in vitro* binding assays that ZO-3 interacts directly with ZO-1 (Haskins *et al.*, 1998). The specific domains responsible for the various binding interactions of ZO-1, ZO-2, and ZO-3 have, in part been defined. ZO-1 and ZO-2 interact via their second PDZ domains (Fanning *et al.*, 1998; Itoh *et al.*, 1999a) and ZO-3 likely interacts with ZO-1 in the same manner (Itoh *et al.*, 1999b). Results presented in Chapter 2 will shed some light on the exact configuration of the protein complex(s) formed between ZO-1, ZO-2, and ZO-3. Furthermore, data shown in Chapter 2 will clarify the direct versus indirect binding of ZO-1 and ZO-2.

*1.4.2. Integral Membrane Protein Binding Interactions*

How the peripherally associated cytoplasmic proteins interact with the transmembrane components of the tight junction has important implications for regulation of paracellular permeability. It is known that both ZO-1 and ZO-3 can directly bind to the cytoplasmic COOH-terminal tail of occludin (Furuse *et al.*, 1994; Fanning *et*

*al.*, 1998; Haskins *et al.*, 1998), and it is thought that the N-terminus of ZO-2 can also interact with occludin (Itoh *et al.*, 1999a), although this was also assayed using whole cell lysates so the interaction may or may not be direct; this will be clarified in Chapter 2. ZO-1 binding to occludin likely occurs through the GUK domain (Fanning *et al.*, 1998). The only binding information known about the claudin family of proteins is that they bind to the first PDZ domain of ZO-1, ZO-2, and ZO-3 via their carboxy-terminal YV sequence (Itoh *et al.*, 1999b). Indirect evidence also suggests that they can form homo- or heterophilic interactions via their extracellular loops, thus linking adjacent cells together (Furuse *et al.*, 1999). The immunoglobulin-like integral membrane protein JAM can dimerize with itself (Bazzoni *et al.*, 2000; Kostrewa *et al.*, 2001), and bind to ZO-1, and cingulin (Bazzoni *et al.*, 2000), as well as with AF-6 (Ebnet *et al.*, 2000). In addition to binding JAM, the cytoplasmic plaque protein cingulin interacts with ZO-1, ZO-2, ZO-3, and also forms homodimers (Cordenonsi *et al.*, 1999), while AF-6 binds to ZO-1 in addition to JAM (Yamamoto *et al.*, 1997).

*1.4.3. Linking Tight Junction Proteins to the Actin Cytoskeleton*

The mechanism through which actin associates with the tight junction was originally proposed as a linkage of actin-ZO-1-occludin based on cosedimentation studies using the COOH-terminal half of ZO-1 (Fanning *et al.*, 1998; Itoh *et al.*, 1997); a region that encompasses the proline-rich domain. However, it is possible that the observed ZO-1-actin interaction is due to unidentified intermediary proteins as actin binding was assayed from whole cell lysates. Itoh *et al.* hypothesized that ZO-2 also interacts with the actin cytoskeleton, as an exogenously expressed COOH-terminal ZO-2 construct

distributes along actin stress fibers in fibroblasts (Itoh *et al.*, 1999a). In Chapter 2 I address other specific mechanisms by which actin associates with the tight junction.

## 1.5. Relationship to the Junctional Complex

The electron microscopic studies of Farquhar and Palade (1963) revealed that the tight junction is part of a larger "junctional complex", consisting of the tight junction, adherens junction, and desmosomes. The tight junction is the apical-most element of the junctional complex, with the adherens junction and desmosomes located more basally. Though these intercellular junctions are distinct morphological entities within the greater junctional complex, there is evidence that there is a considerable amount of cross-talk between the tight junction and adherens junction in particular. The following sections (1.5.1-1.5.3) will provide background information about the adherens junction as a framework for discussing the interplay between both junctions in Section 1.6.

### *1.5.1. Morphology and Physiological Function of the Adherens Junction*

In epithelial and endothelial cells, the adherens junction forms a continuous adhesion belt around the apical circumference of the cells. By electron microscopy the adherens junction appears as closely apposed plasma membranes with a dense cytoplasmic plaque at the cytoplasmic surface (Farquhar and Palade, 1963). As the name suggests, adherens junctions are intercellular junctions that provide adhesion between adjacent cells within the monolayer. The selective cell-cell adhesion provided by adherens junctions is important for tissue remodeling during development, the maintenance of tissue architecture in multicellular organisms, and prevention of tumor metastasis (Gumbiner, 1996).

Like the tight junction, the adherens junction is comprised of integral membrane proteins, and cytoplasmic peripheral membrane proteins that connect the adherens junction to the actin cytoskeleton. The major integral and peripheral membrane adherens junction proteins are summarized in Table 1.2.

*1.5.2. Integral Membrane Proteins of the Adherens Junction: Cadherins*

The integral membrane proteins of the adherens junction are a group of $Ca^{2+}$-dependent cell adhesion molecules called the cadherins. There are different isoforms of cadherins depending on the tissue type examined. In epithelial cells, the predominant cadherin is E-cadherin, while vascular endothelial cells contain VE-cadherin (cadherin-5) (Lampugnani *et al.*, 1995). Cadherins on adjacent cells engage in homophilic adhesive interactions via their extracellular domains, but a second type of cadherin interaction is required to strengthen and stabilize this adhesion. Lateral dimerization of cadherin molecules within the same cell is required to cluster the cadherins on the cell surface (Brieher *et al.*, 1996), and this lateral interaction requires the intracellular juxtamembrane region of cadherin (Yap *et al.*, 1998).

*1.5.3. Peripheral Membrane Proteins of the Adherens Junction: Catenins*

Attachment of the cadherins to the actin cytoskeleton is a requirement for adhesive activity. Adherens junctions contain a plaque of cytoplasmic proteins that include the catenins, which function to link the cadherins to the cytoskeleton. Cadherin is directly bound to β-catenin or plakoglobin (γ-catenin), which in turn binds to the F-actin-binding protein, α-catenin (Rimm *et al.*, 1995). Alternate indirect modes of actin cytoskeleton linkage are also possible; α-catenin can also bind to vinculin (Weiss *et al.*,

Table 1.2. Adherens Junction-Associated Proteins

| **Adherens Junction Proteins** | |
|---|---|
| **Integral Membrane Proteins:** | |
| E-cadherin | (see text) |
| Nectin | Takahashi *et al.*, 1999 |
| **Peripheral Membrane Proteins:** | |
| Catenins: | |
| α–catenin | (see text) |
| β–catenin | (see text) |
| γ–catenin (plakoglobin) | Cowin *et al.*, 1986 |
| δ–catenin | Lu *et al.*, 1999 |
| ARVCF | Mariner *et al.*, 2000 |
| p120 catenin | Shibamoto *et al.*, 1995 |
| α–actinin | Geiger *et al.*, 1981 |
| Vinculin | Geiger *et al.*, 1981 |
| Ponsin | Mandai *et al.*, 1999 |
| AF-6 (afadin) | Mandai *et al.*, 1997 |
| F-actin | (see text) |

1998), α-actinin (Knudsen *et al.*, 1995) and ZO-1 (Itoh *et al.*, 1997), which can all in turn bind to F-actin.

Another catenin that binds to the cytoplasmic portion of cadherins is p120 catenin. Originally identified as a Src substrate (Reynolds *et al.*,1989), p120 catenin was later discovered to contain armadillo repeats and cadherin binding activity (Reynolds *et al.*, 1992). Like β-catenin, p120 catenin binds directly to E-cadherin, although at the juxtamembrane region of the E-cadherin cytoplasmic tail, where it aids in lateral clustering and strengthening of adhesive interactions (Yap *et al.*,1998), but p120 catenin does not interact with α-catenin as does β-catenin (Daniel and Reynolds, 1995). However, p120 catenin also has functions outside of the adherens junction; under certain conditions, p120 catenin can also enter the nucleus (van Hengel *et al.*, 1999) and interact with a transcription factor called Kaiso (Daniel and Reynolds, 1999). The dual roles of both β-catenin and p120 catenin will be discussed in depth in Chapter 3 and Chapter 4, respectively.

## 1.6. Cross-Talk Between the Tight Junction and Adherens Junction

As the tight junction and adherens junction are parts of a greater junctional complex, it would make sense that some aspects of their regulation and assembly might be coordinated. Support for this includes molecular evidence (binding interactions and localization studies), functional cross-talk, and coordinated assembly.

### *1.6.1. Molecular Cross-talk*

One line of evidence for tight junction and adherens junction cross-talk comes from the fact that ZO-1 is expressed in non-tight junction forming cells such as

astrocytes, Schwann cells, and dermal fibroblasts (Howarth *et al.*, 1992), and in these cell types ZO-1 is found at the adherens junction, colocalizing with cadherins (Itoh *et al.*, 1991; Itoh *et al.*, 1993; Howarth *et al.*, 1995). ZO-2 is also found at adherens junctions in nonepithelial cells such as fibroblasts and cardiac muscle cells that lack tight junctions (Itoh *et al.*, 1999a). The mechanism by which ZO-1 and ZO-2 localize to the adherens junction in certain cell types is thought to be via binding to α-catenin (Itoh *et al.*, 1997; Itoh *et al.*, 1999a). ZO-1 has also been shown to bind to AF-6 (afadin), which may be found at either the tight junction or the adherens junction (Mandai et al, 1997; Yamamoto *et al.*, 1997).

*1.6.2. Functional Cross-talk During Assembly*

There is also functional evidence that suggests coordination between the adherens junction and the tight junction, especially during junctional assembly. During junctional assembly ZO-1 is first transiently colocalized with E-cadherin at the adherens junction, and then at later stages is recruited to the tight junction (Rajasekaran *et al.*, 1996). Second, when MDCK cells are incubated with inhibitory antibodies to E-cadherin, not only is adherens junction assembly affected, but tight junction assembly is also disrupted (Gumbiner and Simons, 1986; Gumbiner et al, 1988). Only when E-cadherin relocalizes to the membrane do the cells eventually overcome the inhibitory effect and form junctions. From these results, the authors speculated that E-cadherin mediates an early adhesion event that is a prerequisite for the assembly of both adherens junctions and tight junctions (Gumbiner *et al.*, 1988). Another way of disrupting E-cadherin adhesive activity is to culture MDCK cells in the absence of $Ca^{2+}$ ions. These conditions that disrupt the $Ca^{2+}$-dependent cell adhesion mediated by cadherins also cause the

mislocalization of the tight junction protein ZO-1 (Siliciano and Goodenough, 1988). Calcium removal not only affects the localization of cadherins and ZO-1, but it also causes the TER of the monolayer to drop, indicating that the tight junction barrier has been compromised (Cereijido *et al.*, 1978; Martinez-Palomo *et al.*, 1980). This specific consequence of $Ca^{2+}$ removal is the basis for the "calcium switch" experimental paradigm for analyzing junctional assembly (see Chapter 3).

These lines of evidence suggest that tight junction assembly and function is dependent on a functional adherens junction complex. In Chapter 3 I present evidence that the reverse is also true; disruption of the tight junction can also negatively impact adherens junction assembly, and this indicates that previous notions about the hierarchy of junctional formation require revision.

Up until this point I have focused on morphological and molecular description of the tight junction and provided a brief background on the adherens junction since it is also part of the junctional complex and they can be jointly regulated. Section 1.7 will describe the different ways that the paracellular barrier can be regulated, and how these junctional components are assembled.

## 1.7. Signaling Pathways Converging on the Tight Junction

### *1.7.1. Diverse Signaling Molecules Impact Tight Junction Physiology*

There is a vast and mainly observational collection of data correlating tight junction assembly with intracellular signaling pathways involving molecules such as heterotrimeric G proteins, phospholipase C (PLC), and protein kinase C (PKC). For instance, it was found that transfecting MDCK cells with a constitutively active

heterotrimeric G protein α subunit accelerates tight junction assembly (Denker *et al.*, 1996). Conversely, inhibitors of G protein function such as cholera toxin, pertussis toxin, and $AlF_3$ have been shown to delay tight junction assembly in MDCK cells (Balda *et al.*, 1991). More downstream events such as activation of PLC (artificially via thyrotropin-1 releasing hormone) cause an increase in TER (Balda *et al.*, 1991). PKC activation is also likely involved somehow in regulating tight junction assembly and function. Stimulation of PKC with a diacylglycerol analogue (1,2-dioctanoylglycerol) causes ZO-1 to translocate to the membrane and increased TER even under low calcium conditions (Balda *et al.*, 1993). The importance of PKC phosphorylation events in tight junction assembly is underscored by the observation that PKC can directly phosphorylate ZO-1 *in vitro*, and membrane-associated PKC activity doubles during the junctional assembly process (Stuart and Nigam, 1995). Interestingly, activation of protein kinases is required not only for junctional assembly, but also for junctional disassembly; in one study, cingulin, ZO-1, and E-cadherin did not relocalize away from the membrane after incubation in low calcium if the cells were pretreated with protein kinase C inhibitors (Citi, 1992).

*1.7.2. Role of Phosphorylation Events in Tight Junction Assembly/Regulation*

In keeping with the role of the protein kinase signaling pathways during junctional assembly described in Section 1.7.1, many tight junction proteins can be phosphorylated. However, since the bulk of the data is observational and cannot yet explain the exact mechanism of regulation by phosphorylation, I will just briefly talk about two examples: ZO-1 and occludin. ZO-1 is phosphorylated on serine residues under steady-state conditions (Anderson *et al.*, 1988), and can become tyrosine-

phosphorylated in response to certain extracellular stimuli (e.g., EGF) (Van Itallie *et al.*, 1995). In general, ZO-1 phosphorylation seems to correlate with tight junctional localization; ZO-1 is less phosphorylated in non-tight junction-forming cells such as S180 cells, or in MDCK cells incubated in low calcium (Howarth *et al.*, 1994). Occludin is phosphorylated on serine/threonine residues, and increased phosphorylation of occludin results in its recruitment to the tight junction and resistance to non-ionic detergent extraction (Sakakibara *et al.*, 1997; Wong, 1997), indicating that phosphorylation-dephosphorylation of occludin could be one mechanism for regulating tight junction barrier function.

*1.7.3. Role of Rho Family of Small GTPases in Junctional Assembly*

The Rho family of small GTPases are another group of signal transduction molecules that are gaining acceptance as regulators of tight junction and adherens junction assembly and function. The family members RhoA, Rac, and Cdc42 have classically been assigned roles in regulation of actin dynamics within the cell (Nobes and Hall, 1995). This indicates that these molecules are involved in a huge number of cellular processes that depend on actin cytoskeleton remodeling including cell migration and spreading, cell adhesion, cytokinesis, and cell morphology changes. Because of the close association of F-actin with the junctional complex in epithelial cells, it becomes apparent that these GTPases might play a role in junction physiology. In Chapter 4 I will present data that begin to suggest a molecular link between the tight junction protein ZO-3 and the Rho GTPases.

### 1.8. This Project: Research Rationale

Given the rapid pace of identification of new tight junction proteins, relatively speaking, the knowledge about how these proteins interact and assemble into functional tight junctions is still lacking. The aim of this project was to gain more information about the binding interactions between some tight junction proteins, and more specifically, to begin to elucidate the function of the tight junction protein ZO-3. In Chapter 2 I provide novel information concerning the molecular interactions of ZO-1, ZO-2, ZO-3, and identify that F-actin has multiple possible binding partners at the tight junction. Chapter 3 focuses more specifically on ZO-3 function; how a mutant ZO-3 protein construct perturbs the junctional complex assembly process, and I show some unexpected effects it has on the adherens junction and the actin cytoskeleton. Chapter 4 further explores the effects expressing this mutant tight junction protein has on actin cytoskeleton dynamics within the cell, and discusses a possible link between the tight junction protein ZO-3 and Rho GTPase family signaling pathways. In summary, the data presented in this thesis provide novel information about tight junction assembly and function.

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# CHAPTER 2

## Protein Interactions at the Tight Junction: Actin has Multiple Binding Partners, and ZO-1 Forms Independent Complexes with ZO-2 and ZO-3 [1]

[1] A version of this chapter has been published. Wittchen ES, Haskins J, Stevenson BR. 1999. Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. *J Biol Chem*. 274(49):35179-85. Used with permission from The American Society for Biochemistry and Molecular Biology (ASBMB).

J. Haskins contributed to Figures 2.5 and 2.6.

## 2.1. Introduction

The tight junction acts as a semi-permeable barrier between adjacent cells that keeps the apical and basolateral fluid compartments on opposite sides of the epithelial cell layer separate ("gate function"). The tight junction also acts in the maintenance of apical versus basolateral plasma membrane compositional asymmetry by restricting the movement of lipids and integral membrane proteins within the plane of the membrane ("fence function").

### *2.1.1. Tight Junction-Associated Proteins*

New tight junction proteins are being identified at an exponential rate. Since the identification and characterization of the first tight junction protein ZO-1 (Stevenson *et al.*, 1986; Anderson *et al.*, 1988), a host of other proteins which localize to the tight junction have been identified (refer to Table 1.1). The peripheral membrane proteins ZO-2 (Jesaitis *et al.*, 1994; Beatch *et al.*, 1996) and ZO-3 (Haskins *et al.*, 1998), together with ZO-1, are members of the MAGUK family of proteins (Anderson, 1996) displaying a characteristic multi-domain structure comprised of the PDZ, SH3 and guanylate kinase-like (GUK) domains. ZO-1, ZO-2, and ZO-3, unlike other members of the MAGUK family, each also have an acidic domain, a basic arginine-rich region, and a proline-rich domain. Transmembrane protein components of the tight junction include occludin (Furuse *et al.*, 1993), JAM (Martin-Padura *et al.*, 1998), the claudin family of proteins (Furuse *et al.*, 1998; Morita *et al.*, 1999), CAR (Cohen *et al.*, 2002), and ESAM (Nasdala *et al.*, 2002).

Actin filaments are known to be associated with the tight junction cytoplasmic plaque (Hirokawa and Tilney, 1982; Madara, 1987) and are believed to act in the

regulation of tight junction permeability (Madara *et al.*, 1986; Madara and Pappenheimer, 1987). Investigators have proposed a linkage of actin-ZO-1-occludin as the mechanism by which actin is linked to the tight junction (Itoh *et al.*, 1997; Fanning *et al.*, 1998). However, it is possible that the observed ZO-1-actin interaction is due to unidentified intermediary proteins as actin binding was assayed from whole cell lysates in these experiments. Itoh *et al.*. have hypothesized that ZO-2 also interacts with the actin cytoskeleton, as an exogenously expressed COOH-terminal ZO-2 construct distributes along actin stress fibers in fibroblasts (Itoh *et al.*, 1999a). Evidence exists that occludin, and not other transmembrane elements of the tight junction, functions in the linkage between the tight junction and the actin cytoskeleton (Kojima *et al.*, 1999).

*2.1.2. Binding Interactions*

More information is becoming available regarding binding interactions among tight junction-associated proteins (refer to Chapter 1 for complete summary). How the tight junction MAGUK proteins ZO-1, ZO-2, and ZO-3 interact with each other, and other binding partners is of obvious interest considering their proposed scaffolding role. ZO-1, ZO-2 and ZO-3 can be coimmunoprecipitated from MDCK cells (Balda *et al.*, 1993; Jesaitis *et al.*, 1994; Haskins *et al.*, 1998). We have previously shown via *in vitro* binding assays that ZO-3 interacts directly with ZO-1 and the cytoplasmic COOH-terminal tail of occludin, but does not bind ZO-2 (Haskins *et al.*, 1998). Based on immunoprecipitation of protein constructs from transfected cells, recent reports suggest that ZO-1 and ZO-2 interact with each other via their second PDZ domains (Fanning *et al.*, 1998; Itoh *et al.*, 1999a). However, these binding studies were also performed with whole cell lysates, and the possibility that the interaction is indirect and mediated by other proteins cannot be

excluded. It is known that ZO-1 can directly bind to the cytoplasmic COOH-terminal tail of occludin (Fanning *et al.*, 1998; Furuse *et al.*, 1994) and the Ras-effector molecule AF-6 (Yamamoto *et al.*, 1997). Recent data suggest that the N-terminus of ZO-2 also interacts with the COOH-terminal tail of occludin (Itoh *et al.*, 1999a), although again, this was assayed using whole cell lysates.

### *2.1.3. Chapter Summary*

Defining how the molecular components of the tight junction interact will provide information crucial to understanding tight junction physiology. Here we define the interactions between ZO-1, ZO-2, ZO-3, occludin, and F-actin through the use of purified proteins and *in vitro* binding assays. Our data show that the interactions between the actin cytoskeleton and junctional components are more complex than anticipated, consisting of multiple possible linkage mechanisms involving ZO-2, ZO-3, and occludin. Low speed sedimentation analyses demonstrated that neither ZO-2, ZO-3, nor occludin act as F-actin crosslinking proteins, and further evidence indicates that these proteins do not bind to actin filament ends. In addition, our *in vitro* binding results reveal that previous views on the existence of a three-member complex of ZO-1, ZO-2, and ZO-3 require revision. The experiments outlined in this chapter provide information regarding direct binding interactions among tight junction-associated proteins. The emerging complexity of protein-protein interactions at the tight junction has both functional and regulatory implications.

## 2.2. Experimental Procedures

### *2.2.1. Protein Expression*

Recombinant protein expression and purification was performed according to previously published methods (Haskins *et al.*, 1998). In brief, full length 6-histidine tagged ZO-3 (pHTc/ZO-3) and ZO-2 (pHTb/ZO-2) proteins were each expressed in Sf9 insect cells using a Baculovirus eukaryotic expression system (GIBCO BRL, Gaithersburg, MD). Empty pFastBac vector (pHTc), coding for the 6-histidine tag plus 36 nonspecific amino acids served as negative control. Protein from Sf9 cell lysate was purified on Probond nickel resin (Invitrogen Corp.), eluted with 300-500 mM imidazole, and used in binding assays or cosedimentation experiments. JShak2, a 6-histidine tagged jellyfish Shaker-type $K^+$-channel peptide (amino acids 402-476; ref 28, a gift from Dr. Warren Gallin, University of Alberta) in the pRSET expression vector (Invitrogen Corp., San Diego, CA) served as a negative control for ZO-2 and ZO-3/actin cosedimentation studies. GST-occludin cDNA encoding the cytoplasmic COOH-terminal tail (amino acids 358-505) of chicken occludin (Furuse *et al.*, 1993) in pGEX-2T (a gift from Drs. Jim Anderson and Alan Fanning, Yale University), and GST alone were expressed according to manufacturer protocol (Pharmacia Biotech, Inc.). GST fusion proteins were purified on glutathione-Sepharose 4B resin (Pharmacia Biotech, Inc.) and eluted with 50 mM reduced glutathione. [$^{35}$S] methionine-labeled *in vitro* transcribed/translated ZO-1 was prepared with the TNT® T7-coupled reticulocyte system (Promega Corp.) using as template full length human ZO-1 cDNA (obtained from Drs. Anderson and Fanning) in pBluescript SK+ under control of the T7 promoter.

*2.2.2. Antibodies*

The following antibodies were used: rat anti-ZO-1 mAb R40.76 (Anderson *et al.*, 1988), rabbit anti-ZO-2 polyclonal Ab R9989 (Jesaitis *et al.*, 1994), guinea pig anti-ZO-3 (Haskins *et al.*, 1998) and rabbit anti-VSVG (a gift from Carolyn Machamer, Johns Hopkins University, Baltimore, MD). The anti-ZO-3 antibody does not cross-react with ZO-1 or ZO-2. A rabbit anti-occludin antibody (raised against amino acids 255-510) (Wong and Gumbiner, 1997; a gift from Drs. Alpha Yap and Barry Gumbiner) was used for immunoblot detection of both recombinant occludin and GST alone as it was raised against a GST fusion protein. A second rabbit anti-occludin antibody raised against the most COOH-terminal 150 amino acids (Zymed Laboratories Inc., San Francisco, CA) was used for immunofluorescence experiments. A cocktail of anti-tetraHis and anti-pentaHis antibodies (Qiagen, Valencia, CA) was used to recognize the 6-histidine tag of the JShak2 fusion protein. HRP-conjugated goat anti-rabbit (Bio-Rad Laboratories, Hercules, CA), HRP-conjugated goat anti-guinea pig (Jackson ImmunoResearch Laboratories, Inc.), and rhodamine-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, Inc.) were used as secondary antibodies for immunoblots and indirect immunofluorescence. Immunoblot detection via ECL (Amersham Corp., Arlington Heights, IL) was performed according to manufacturer protocols.

*2.2.3. Actin Cosedimentation Experiments and Low Speed Sedimentation Analysis*

Actin cosedimentation experiments were performed according to modification of the methods of Fanning *et al.* (Fanning *et al.*, 1998). Briefly, lyophilized G-actin (>95% pure grade) (Cytoskeleton Inc., Denver, CO) was diluted to 10 mg/ml with distilled water, so that buffer conditions were then 10 mM Tris-HCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, 1%

dextran, 5% sucrose. Further dilution with actin binding buffer (10 mM Imidazole pH 7.2, 75 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT) to a final actin concentration of 2.5 mg/ml was followed by 30 min incubation on ice to initiate polymerization. Polymerized F-actin was stabilized by adding phalloidin (Sigma Chemical Co., St. Louis, MO) to 25 μg/ml (Fanning et al., 1998). Purified ZO-2, ZO-3, JShak2, GST-occludin, or GST were dialyzed into actin binding buffer containing 0.1% Triton X-100 to inhibit aggregate formation, then pre-centrifuged at 100,000 *g* for 30 min. F-actin binding was assayed in 100 μl reactions containing a final concentration of 0.25 mg/ml F-actin (or actin binding buffer alone), and pre-centrifuged purified test protein. Each reaction was incubated at room temperature for 30 min before spinning 30 min at 100,000 *g*. The supernatant fraction was removed and pellets were resuspended in an equal volume of gel sample buffer to maintain equivalent stoichiometry between supernatant and pellet fractions. Samples were run on SDS-PAGE and processed for immunoblotting. For actin cosedimentation assays using sheared actin, shearing was accomplished by pipetting the F-actin solution 20 times through a narrow bore pipet tip. Shearing of F-actin was confirmed by falling ball viscometry (MacLean-Fletcher and Pollard, 1980) to measure relative viscosity of unsheared versus sheared F-actin solutions. Test proteins were added to unsheared or sheared F-actin immediately after shearing.

Low speed sedimentation analysis was performed according to established methods (Espindola *et al.*, 1992). Briefly, 100 μl reactions containing 0.25 mg/ml actin alone, or in the presence of purified ZO-2, ZO-3, occludin, or a 3 μg/ml solution of poly-L-lysine were incubated for 30 min at room temperature before centrifugation at 10 000 *g* in a benchtop microcentrifuge for 20 min. The supernatants were removed, and the pellet

fractions were resuspended in an equal volume of gel sample buffer. Supernatant and pellet fractions were run on SDS-PAGE and stained with Coomassie brilliant blue to detect F-actin.

*2.2.4. Cytochalasin D Experiments*

Control MDCK (strain II) and MDCK cells expressing full length exogenous VSVG-tagged ZO-3 (MDCK/Z3) (Haskins *et al.*, 1998) were grown on rat tail collagen-coated coverslips to near confluency. Cytochalasin D (cD) experiments were performed according to established methods (Stevenson and Begg, 1994). Briefly, coverslips were treated for 60 min with either 2 μg/ml cD (from a 5 mg/ml DMSO stock) or an equal concentration of DMSO alone as a negative control. Cells were then immediately fixed with 2.5% paraformaldehyde and permeabilized with 0.2% Triton X-100 in TBS and fixed for immunofluorescence as described (Howarth *et al.*, 1992). FITC-conjugated phalloidin (Sigma) was used to stain F-actin. Coverslips were viewed on a Zeiss Axioskop (Carl Zeiss, Inc., Oberkochen, Germany). For costaining experiments, control coverslips stained for ZO-2, ZO-3, occludin, or actin alone showed no visible fluorescence from either fluorochrome when viewed with the opposite filter set (data not shown).

*2.2.5. Direct Binding: Affinity Chromatography*

*ZO-2/ZO-1 Binding:* Negative control peptide from cells containing pHTc alone, or purified ZO-2 dialyzed against PBS was bound to Probond resin and washed with phosphate wash buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 25 mM Imidazole) with 0.2% Tween 20, 0.2% Triton X-100, and protease inhibitors (1 μg/ml aprotinin, 0.2 mM PMSF, 1 μg/ml chymostatin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Protein-containing resin was incubated with the completed ZO-1 *in vitro* transcription/translation reaction diluted

so that final binding conditions were 140 mM KCl, 25 mM Imidazole, 10 mM HEPES, 0.1% Tween 20, 0.1% Triton X-100, and protease inhibitors. Binding proceeded overnight at 4°C, followed by washing with phosphate wash buffer with 0.1% Triton X-100 and 0.1% Tween 20. Washed resin was resuspended in gel sample buffer, boiled, and bound protein complexes were run on SDS-PAGE. Enhancement of $^{35}S$ signal was accomplished by sequential incubation of the gel in 40% methanol/10% acetic acid (30 min) and 1M salicylic acid solution (30 min). The gel was dried and exposed to Fuji RX film (Fuji Photo Film Co. Ltd., Japan).

*ZO-2/Occludin Binding:* GST-occludin or GST alone was purified on glutathione-Sepharose 4B beads, and Probond-purified ZO-2 was added. Final binding conditions were 140 mM KCl, 10 mM HEPES pH 7.5, 1mM $MgCl_2$, 0.1% Triton X-100, and 0.1% Tween 20. Binding was allowed to proceed overnight at 4°C. After washing with the same buffer, bound protein was eluted by boiling the beads in gel sample buffer, resolved on SDS-PAGE, electrophoretically transferred, and immunoblotted with corresponding antibodies.

*2.2.6. Immunoprecipitation Experiments*

*Low Stringency Immunoprecipitation:* MDCK or MDCK/Z3 cells were grown and metabolically labeled as described previously (Anderson *et al.*, 1988; Stevenson *et al.*, 1988). Low stringency immunoprecipitations, defined as conditions that maintain some protein-protein interactions, were performed according to previously published techniques (Gumbiner *et al.*, 1991; Jesaitis *et al.*, 1994; Haskins *et al.*, 1998). Confluent monolayers of MDCK cells were solubilized as described, and centrifuged at 13,000 *g* for 10 min at 4 °C to remove cell debris. In experiments requiring equivalent starting

material, total protein concentration of the supernatant fraction was determined by BCA protein assay (Pierce, Rockford, IL) prior to immunoprecipitation. This supernatant was then immunoprecipitated with either anti-ZO-1 mAb followed by goat anti-rat IgG coupled to cyanogen-bromide-activated Sepharose 4B (Pharmacia Biotech, Inc., Piscataway, NJ), anti-ZO-2 followed by protein A-Sepharose, or anti-ZO-3 followed by protein A-Sepharose. Sepharose beads were washed according to procedures of Pasdar and Nelson (Pasdar and Nelson, 1989) and solubilized in gel sample buffer before running on SDS-PAGE and processing for autoradiography.

*Sequential Immunoprecipitation:* Low stringency immunoprecipitation followed by high stringency immunoprecipitation was performed with modification of previously published techniques (Li *et al.*, 1998). Metabolically labeled MDCK cells were first immunoprecipitated under low stringency conditions (described above), and the resulting immune complexes were eluted from the Sepharose beads under high stringency conditions (Stevenson *et al.*, 1994) by boiling in 1% SDS, 10 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF to dissociate protein complexes. The supernatants were adjusted to 0.2% SDS by dilution with 50 mM Tris pH 7.4, 190 mM NaCl, 10 mM EDTA, 2.9% Triton X-100, before reimmunoprecipitating a second time with the indicated antibodies.

## 2.3 Results

### *2.3.1. Actin-Tight Junction Linkages*

Although actin filaments have been observed at the tight junction (Hirokawa and Tilney, 1982; Madara, 1987) and have been implicated in the regulation of junction permeability (Madara *et al.*, 1986; Madara and Pappenheimer, 1987), the linkage through

which this interaction is mediated has not been unequivocally demonstrated. Previous work (Fanning *et al.*, 1988; Itoh *et al.*, 1997) suggested an association between actin and ZO-1, although these experiments did not rule out the possibility that the interaction occurs via intermediary proteins.

We performed a series of actin cosedimentation assays to test directly the F-actin binding ability of other tight junction proteins, using purified recombinant ZO-2 and ZO-3. We also tested the F-actin binding ability of occludin to determine if linkage to the cytoskeleton is mediated exclusively by peripheral membrane proteins, or if actin can bind directly to a transmembrane element. The actin cosedimentation assay is based on the observation that polymerized actin filaments are readily pelleted by high-speed centrifugation (Figure 2.1, part A). When ZO-2 (Figure 2.1, part B) is added to the reaction, there is a significant shift of ZO-2 from the supernatant to the pellet fraction only in the presence of F-actin. A negative control protein ($(His)_6$-tagged JShak2 fusion protein) did not cosediment with F-actin (Figure 2.1, part B), indicating that the ZO-2/actin association is not due to the 6-histiding tag binding F-actin. Furthermore, ZO-2 still cosedimented with F-actin after the 6-histidine tag had been removed by Factor Xa cleavage (data not shown). ZO-3 was similarly found to cosediment with F-actin (Figure 2.1, part C). Interestingly, GST-occludin was also found to cosediment specifically with F-actin, while the GST alone negative control peptide did not (Figure 2.1, part D). ZO-2, ZO-3, and occludin proteins also cosedimented with F-actin when spun through a 10% glycerol cushion (data not shown), further indicating that the observed interactions with

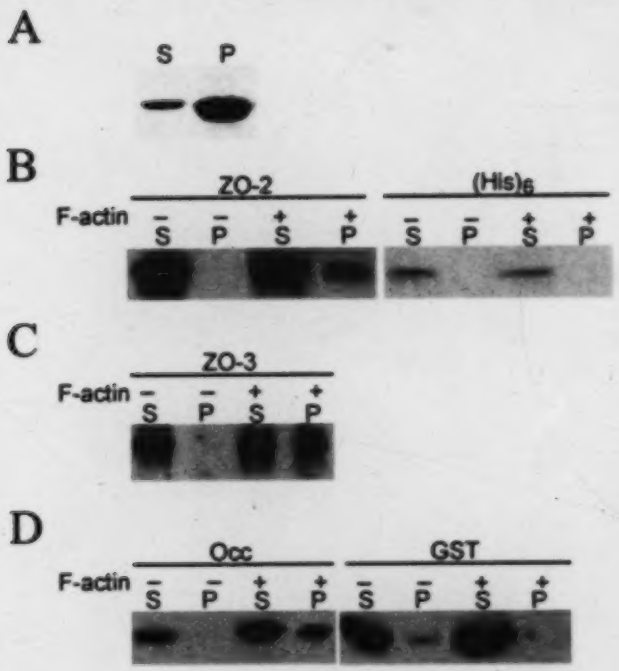



**Figure 2.1. Purified, recombinant ZO-2, ZO-3, and occludin specifically cosediment with F-actin.** (A) Coomassie blue-stained gel of phalloidin-stabilized F-actin alone centrifuged at 100,000 *g*. Equivalent aliquots of supernatant and pellet fractions were loaded. Polymerized F-actin is found almost entirely in the pellet. (B) ZO-2 binds F-actin specifically. ZO-2 or a negative control 6-histidine tagged protein were incubated in the presence (+) or absence (-) of F-actin, centrifuged at 100,000 *g*, and equivalent supernatant and pellet fractions were loaded and immunoblotted for ZO-2 or the 6-histidine tag. ZO-2 is associated with the pellet fraction only in the presence of F-actin. The 6-histidine tagged protein JShak2 does not cosediment with actin. (C) ZO-3 binds F-actin. ZO-3 was incubated in the presence (+) or absence (-) of F-actin, treated as in part B, and immunoblotted for ZO-3. ZO-3 is associated with the pellet fraction only in the presence of F-actin. (D) The C-terminus of occludin binds F-actin. GST-occludin or GST alone were incubated in the presence (+) or absence (-) of F-actin, treated as in B and C, and immunoblotted for GST-occludin. GST-occludin is associated with the pellet fraction only in the presence of F-actin. GST alone does not cosediment with actin.

actin are specific and are not due to trapping by actin filaments. Combined, these data suggest that F-actin is capable of specifically binding multiple tight junction proteins.

F-actin binding proteins can be classified based on the type of interaction with actin. For example, there are F-actin end-binding (capping) proteins such as CapZ (Casella *et al.*, 1987), side-binding proteins (non-crosslinking) such as tropomyosin (Cote and Smillie, 1981), and crosslinking proteins such as α-actinin (Blanchard *et al.*, 1989). We tested if ZO-2, ZO-3 and occludin bind to actin filament ends by utilizing actin cosedimentation experiments with unsheared versus sheared actin filaments. Shearing creates shorter actin filaments and therefore more actin ends available for binding. Confirmation of shearing was done by falling ball viscometry, which is a means of measuring relative viscosity of a solution. Our shear conditions resulted in a significant decrease in viscosity. We observed a fall rate of 0.69 cm/s ± 0.035 for unsheared F-actin and 0.90 cm/s ± 0.025 for sheared F-actin. The amount of pelletable F-actin did not change after shearing (Figure 2.2, part A). We conclude that these shear conditions result in a greater number of F-actin ends. When ZO-2, ZO-3, or occludin was incubated with either unsheared (-) or sheared (+) actin filaments, there was no change in the amount of test protein found in the pellet (Figure 2.2, part A), indicating that these proteins do not preferentially bind to F-actin ends. F-actin side-binding proteins can act as crosslinkers if they have the ability to bind to more than one actin filament simultaneously. To test ZO-2, ZO-3, and occludin for actin filament crosslinking ability, we performed low speed sedimentation assays by centrifugation at 10 000 *g* for 20 min (Espindola *et al.*, 1992). Under these conditions, the majority of single actin filaments remain in the

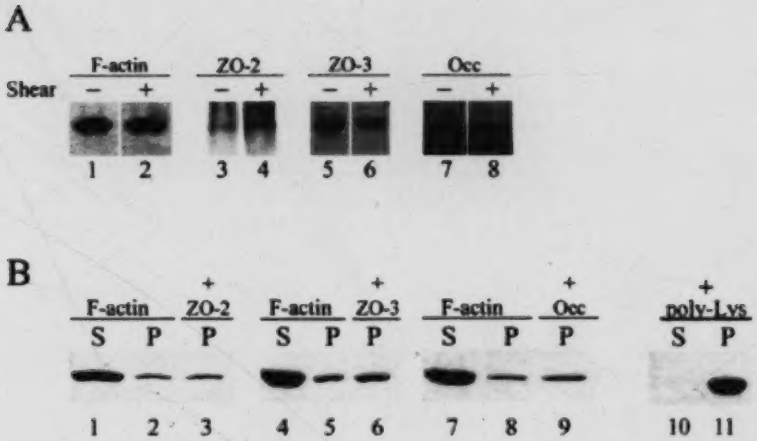



**Figure 2.2. ZO-2, ZO-3, and occludin do not bind actin filament ends, and do not act as actin crosslinking proteins.** (A) Pellet fractions of actin cosedimentation experiments using unsheared (-) versus sheared (+) F-actin alone, or F-actin in the presence of ZO-2, ZO-3, or occludin. Shearing creates shorter filaments and therefore generates more free ends but does not alter the amount of F-actin in the pellet fraction (lanes 1-2) visualized by Coomassie blue staining. The amount of ZO-2, ZO-3, or occludin in the pellet fractions does not increase in the presence of sheared F-actin (lanes 4, 6, and 8) compared to unsheared F-actin (lanes 3, 5, and 7), indicating that these proteins do not bind to actin filament ends. Pellet fractions were immunoblotted with anti-ZO-2 (lanes 3-4), anti-ZO-3 (lanes 5-6) and anti-occludin (lanes 7-8). (B) Low speed sedimentation assay to test F-actin crosslinking ability of ZO-2, ZO-3 and occludin. F-actin was incubated alone (lanes 1-2, 4-5, and 7-8) or with ZO-2 (lane 3), ZO-3 (lane 6) or occludin (lane 9), centrifuged at low speed (10 000 *g*), and visualized by Coomassie staining of supernatant (S) and/or pellet (P) fractions. The majority of F-actin is found in the supernatant under these conditions (lane 1, 4, and 7), compared with the pellet fraction (lane 2, 5, and 8). When ZO-2, ZO-3, or occludin is added, the amount of F-actin in the pellet is unchanged, indicating that neither ZO-2, ZO-3, nor occludin has any detectable F-actin crosslinking activity. As a positive control (lanes 10-11), F-actin was incubated with poly-L-lysine, centrifuged at 10 000 g, and visualized by Coomassie staining of supernatant and pellet fractions. Cross-linked F-actin is found entirely in the pellet under these conditions.

supernatant (Figure 2.2, part B), whereas cross-linked actin filament networks readily pellet (lanes 10 and 11). Incubating ZO-2, ZO-3, or occludin with F-actin did not cause an increase in the amount of F-actin pelleted under these conditions (Figure 2.2, part B), suggesting that these proteins do not act to cross-link or bundle F-actin.

While the cosedimentation experiments indicate that ZO-2, ZO-3, and occludin bind F-actin directly *in vitro*, we further explored if these interactions were functionally relevant *in vivo*. Earlier work showed that treating cells with the actin-disrupting drug cytochalasin D (cD) causes the continuous apical actin belt to reorganize into focal aggregates along cell boundaries, and that these aggregates colocalize with aggregates of ZO-1 (Stevenson and Begg, 1994). A corresponding increase in paracellular permeability was also observed in the cD-treated monolayers in this study. There are varying reports on the mechanism of action for cD; it may bind to the barbed (faster-growing) end of actin, thus "capping" the barbed end of actin and preventing G-actin monomers from being added, or it may act to sever actin filaments (Hartwig and Stossel, 1979). In either case, cD treatment disrupts the organization of actin-filaments. To investigate if ZO-2, ZO-3, or occludin also associated with actin filaments *in vivo*, cD-treated or control monolayers were costained for actin and either ZO-2, ZO-3, or occludin. Staining of MDCK monolayers with FITC-conjugated phalloidin (Figure 2.3, part A) reveals a continuous apical actin belt in the same focal plane as the tight junction. ZO-2, ZO-3, and occludin (Figure 2.3, parts B, C, and D) show continuous staining along cell borders typical of these tight junction proteins (Jesaitis *et al.*, 1994; Haskins *et al.*, 1998; Furuse *et al.*,

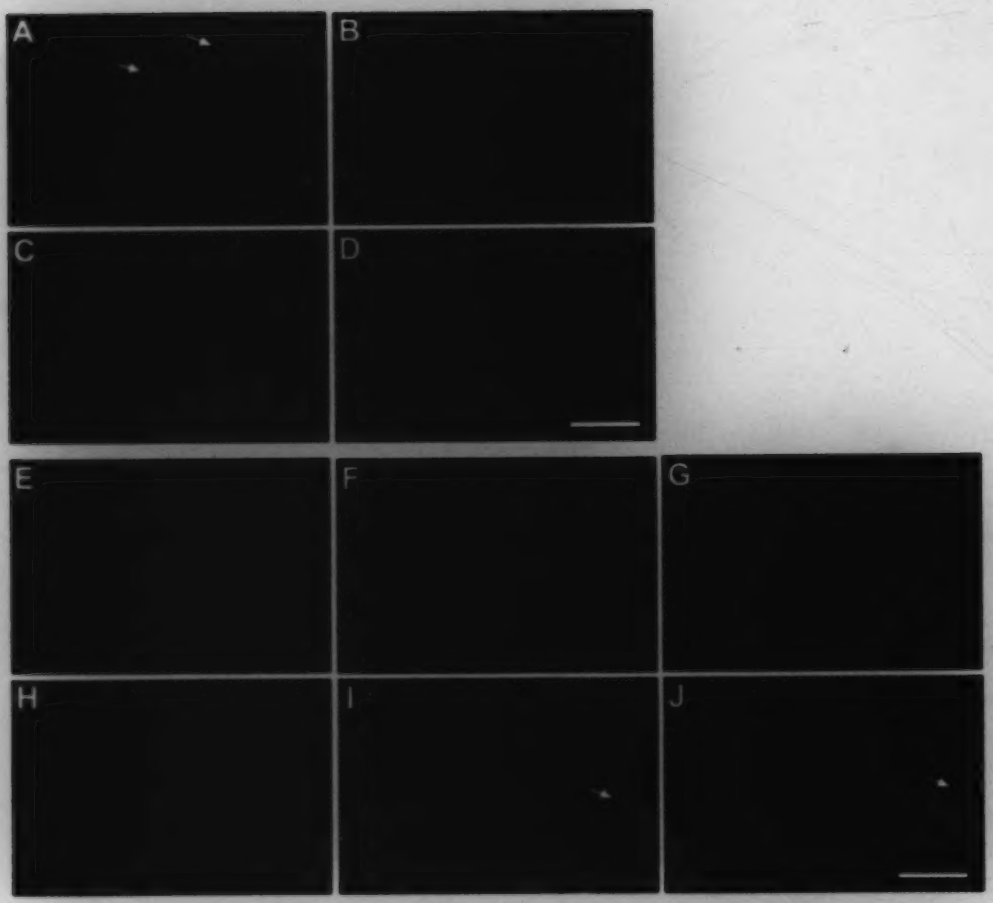



**Figure 2.3. Cytochalasin D treatment of MDCK cells induces focal aggregation of tight junction proteins which colocalize with disrupted actin.** (A) Untreated cells stained with FITC-phalloidin show a continuous ring of apical actin staining at cell borders (arrows) when viewed at the level of the tight junction. More basally located stress fibers are visible. (B-D) Untreated cells stained for ZO-2 (B), ZO-3 (C), or occludin (D) similarly show continuous junctional staining along cell borders. The nuclear staining seen with the anti-occludin antibody is an artifact of paraformaldehyde fixation, as it is not seen when cells are fixed with methanol (data not shown). Bar A-D: 30 μm. (E-J) Cells treated with 2 μg/ml cytochalasin D costained for ZO-2 (E), ZO-3 (F), or occludin (G) and actin (H, I, J). ZO-2, ZO-3, and occludin are reorganized into discontinuous focal aggregates around the cell perimeter which colocalize with similar aggregates of actin. Bar E-J: 15 μm.

1993). When cells are treated with cD and costained for actin (Figure 2.3, parts H, I, and J) and ZO-2 (Figure 2.3, part E), ZO-3 (Figure 2.3, part F), or occludin (Figure 2.3, part G), the continuous junctional staining pattern of tight junction proteins seen in control cells was found to be reorganized into aggregates which colocalize with similar aggregates of actin. The *in vivo* colocalization of ZO-2, ZO-3, and occludin with actin under conditions where tight junction integrity is compromised indicates that the *in vitro* binding interactions identified above also play a role *in vivo*.

*2.3.2. Binding Interactions Involving Occludin and ZO-1, ZO-2, and ZO-3*

As it was shown previously that ZO-1 (Fanning *et al.*, 1998; Furuse *et al.*, 1994) and ZO-3 (Haskins *et al.*, 1998) bind to the COOH-terminal, cytoplasmic tail of occludin, we tested whether ZO-2 shares this ability. Itoh *et al.*. (Itoh *et al.*, 1999a) provided data which indicated that ZO-2 has occludin binding capability; however, the binding assays were performed with whole cell lysates, so the possibility that this interaction is mediated by a third protein could not be excluded. We tested for direct binding of ZO-2 to occludin by incubating purified recombinant ZO-2 with either resin-immobilized GST-occludin or GST alone. Figure 2.4(A) shows that occludin specifically retains ZO-2, while GST alone does not. As an additional negative control, a 6-histidine tagged Rubella virus capsid protein (a gift from Christian Oker-Blom, VTT Biotechnology and Food Research, Helsinki, Finland) did not bind to occludin under identical conditions (data not shown), showing that the occludin construct does not have non-specific binding capabilities, and that the interaction with ZO-2 was not due to the 6-histidine tag.

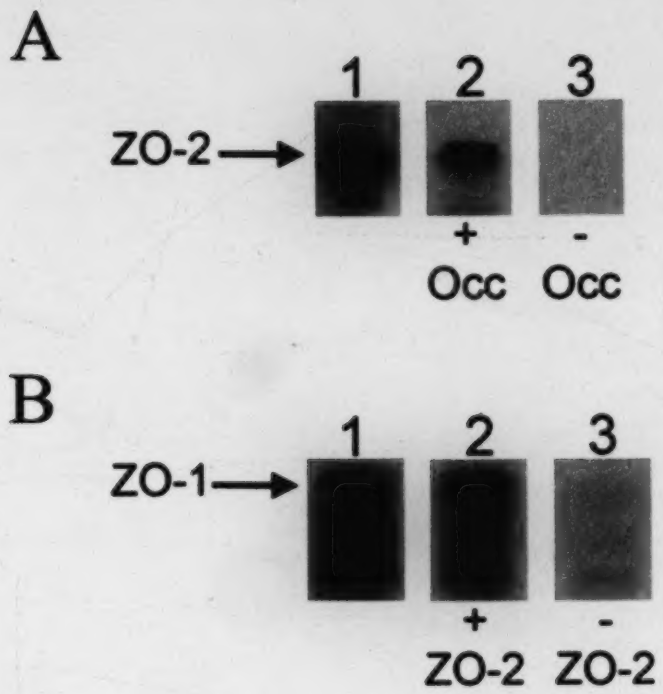



**Figure 2.4. ZO-2 directly binds both occludin and ZO-1.** (A) Purified recombinant ZO-2 (lane 1) was incubated with affinity resin containing the COOH-terminal, cytoplasmic domain of occludin as a GST-fusion protein (lane 2) or GST alone (lane 3). Bound material was eluted, subjected to SDS-PAGE, and immunoblotted with anti-ZO-2. ZO-2 is retained by GST-occludin but not by GST alone. (B) $^{35}$S-labeled *in vitro* translated ZO-1 (lane 1) was incubated with affinity resin containing either ZO-2 (lane 2) or negative control 6-histidine tagged peptide (lane 3). Bound material was eluted, subjected to SDS-PAGE and autoradiography. ZO-1 is specifically retained by ZO-2 but not the negative control polypeptide.

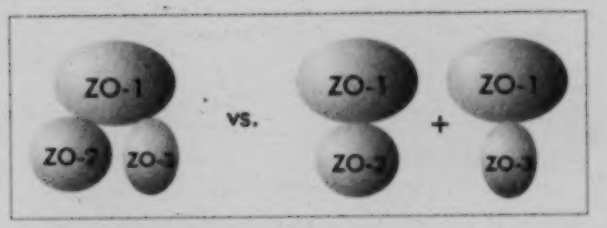
Up to this point, the experimental evidence provided for a binding interaction between ZO-1 and ZO-2 (Fanning *et al.*, 1998; Itoh *et al.*, 1999a) does exclude the possibility that a third unidentified protein is acting as a bridging protein. In the present study, purified recombinant ZO-2 or negative control peptide immobilized on affinity resin was incubated with *in vitro* translated ZO-1, and bound complexes were resolved by SDS-PAGE and autoradiography to detect $^{35}$S-labeled ZO-1. Figure 2.4(B) shows that the ZO-2 affinity column retains ZO-1 while the negative control peptide does not. We previously confirmed the identity of the transcription/translation product shown in lanes 1 and 2 (Figure 2.4) as ZO-1 by immunoblot (Haskins *et al.*, 1998). As a negative control, transcription/translation with empty vector (pBluescript SK+) results in no visible products (data not shown). The faster-migrating multiple bands visible in this sample likely result from proteolytic degradation or incomplete transcription due to the large size of the ZO-1 transcript (>5 Kb). This finding confirms that ZO-1 and ZO-2 bind directly to each other.

*2.3.3. Nature of the ZO-1/ZO-2/ZO-3 Complex*

The finding that ZO-1 binds directly to both ZO-2 (Figure 2.4), and ZO-3 (Haskins *et al.*, 1998), while ZO-2 and ZO-3 do not interact directly (Haskins *et al.*, 1998), makes it possible that ZO-1 may act as a bridging molecule, mediating the interaction of ZO-1, ZO-2, and ZO-3 into the observed coimmunoprecipitating heterotrimeric complex (Balda *et al.*, 1993; Jesaitis *et al.*, 1994; Haskins *et al.*, 1998). Alternatively, available data are also consistent with the presence of distinct ZO-1/ZO-2 and ZO-1/ZO-3 complexes (Figure 2.5, part A). In order to discern between these alternatives, we performed a series of low stringency immunoprecipitations using anti-ZO-1, anti-ZO-2, or anti-ZO-3

**Figure 2.5. Independent ZO-1/ZO-2 and ZO-1/ZO-3 complexes exist *in vivo*.** (A) Schematic representation of two possible configurations involving ZO-1, ZO-2, and ZO-3 that can explain existing immunoprecipitation and *in vitro* binding results. (B) Low stringency anti-ZO-1, anti-ZO-2, or anti-ZO-3 immunoprecipitations (Low IP) from metabolically labeled MDCK cell extracts. Anti-ZO-1 (lane 1) immunoprecipitates ZO-1 (215 kD), ZO-2 (160 kD) and ZO-3 (130 kD). Anti-ZO-2 immunoprecipitates ZO-1 and ZO-2 but not ZO-3 (lane 2). Anti-ZO-3 immunoprecipitates ZO-1 and ZO-3 but not ZO-2 (lane 3). Molecular mass markers at right (kD). (C) Sequential immunoprecipitations. Low stringency immunoprecipitations (equivalent aliquots from part B) were reimmunoprecipitated under high stringency conditions (High IP) with the antibodies indicated. The absence of ZO-3 in the low stringency anti-ZO-2 immunoprecipitate (lane 1), and the absence of ZO-2 in the low stringency anti-ZO-3 immunoprecipitate (lane 2) is confirmed. Arrows indicate the positions of ZO-2 and ZO-3 migration. (D) Control sequential immunoprecipitations showing that ZO-2 and ZO-3 can be successfully reimmunoprecipitated by this technique if present.

A


ZO-1
ZO-2
ZO-3
vs.
ZO-1
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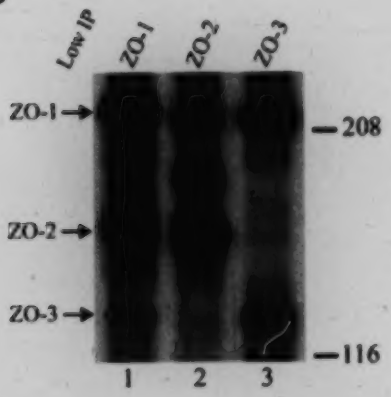
+
ZO-1
ZO-3

B


Low IP
ZO-1
ZO-2
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ZO-1
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208
116
1
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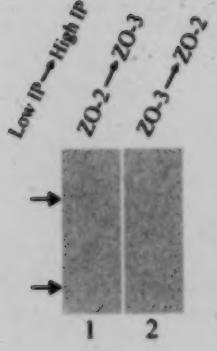
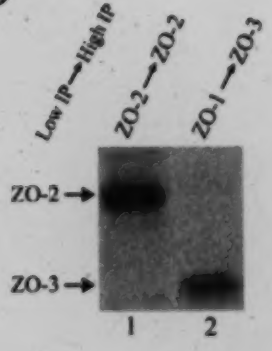
3

C


Low IP→High IP
ZO-2→ZO-3
ZO-3→ZO-2
1
2

D


Low IP→High IP
ZO-2→ZO-2
ZO-1→ZO-3
ZO-2
ZO-3
1
2

antibodies to identify coimmunoprecipitating proteins (Figure 2.5, part B). As shown previously (Gumbiner *et al.*, 1991; Balda *et al.*, 1993; Jesaitis *et al.*, 1994; Haskins *et al.*, 1998), both ZO-2 and ZO-3 coimmunoprecipitate with anti-ZO-1 (Figure 2.5, part B, lane 1). This particular coimmunoprecipitation was the means by which ZO-2 (Gumbiner *et al.*, 1991; Jesaitis *et al.*, 1994) and ZO-3 (Balda *et al.*, 1993; Haskins *et al.*, 1998) were identified and characterized. When a low stringency immunoprecipitation is performed using anti-ZO-2 (Figure 2.5, part B, lane 2), ZO-1 coimmunoprecipitates but ZO-3 does not. The unidentified protein migrating at ~180 kD between ZO-1 and ZO-2 is believed to be a non-specific band related to the rabbit anti-ZO-2 antibody used (Jesaitis *et al.*, 1994). When anti-ZO-3 is used as the immunoprecipitation antibody under low stringency conditions, ZO-1 again coimmunoprecipitates but ZO-2 does not (Figure 2.5, part B, lane 3). To confirm the absence of ZO-3 and ZO-2 from the material shown in lanes 2 and 3 of Figure 2.5 (B) respectively, low stringency immunoprecipitations using anti-ZO-2 and anti-ZO-3 were followed by high stringency immunoprecipitations with anti-ZO-3 and anti-ZO-2 antibodies (Figure 2.5, part C). No ZO-3 is detected in the anti-ZO-2 low stringency immunoprecipitation (Figure 2.5, part C, lane 1), and no ZO-2 is found in the anti-ZO-3 low stringency immunoprecipitation (Figure 2.5, part C, lane 2). To demonstrate the efficacy of this sequential immunoprecipitation approach, Figure 2.5 (D) shows that ZO-2 can be sequentially immunoprecipitated from the anti-ZO-2 low stringency immunoprecipitation (Figure 2.5, part D, lane 1). Similarly, ZO-3 can be sequentially immunoprecipitated from a low stringency anti-ZO-1 immunoprecipitation (Figure 2.5, part D, lane 2). Combined, these data indicate that two distinct complexes exist at the tight junction: ZO-1/ZO-2 and ZO-1/ZO-3.

To further corroborate the existence of two separate complexes involving ZO-1, ZO-2, and ZO-3, MDCK cells overexpressing an exogenous, full-length ZO-3 construct (MDCK/Z3; Haskins *et al.*, 1998) were examined. MDCK/Z3 cells express significantly more ZO-3 than parental cells (Figure 2.6, part A). When ZO-1 is immunoprecipitated from MDCK/Z3 cell extracts under low stringency conditions, less ZO-2 coimmunoprecipitates from MDCK/Z3 cells (Figure 2.6, part B, lane 1) than from parental MDCK cells (Figure 2.6, part B, lane 2). This indicates that in cells overexpressing ZO-3, less ZO-1 is associating with ZO-2, and ZO-1/ZO-3 becomes the predominant complex. Conversely, when low stringency immunoprecipitations with anti-ZO-2 are performed, less ZO-1 coimmunoprecipitates from MDCK/Z3 cells (Figure 2.6, part B, lane 3) than from parental MDCK cells (Figure 2.6, part B, lane 4), with no ZO-3 coimmunoprecipitating from either cell type. Finally, as expected, there is more ZO-1 coimmunoprecipitated by anti-ZO-3 in MDCK/Z3 cells (Figure 2.6, part B, lane 5) than in parental MDCK cells (Figure 2.6, part B, lane 6), and no ZO-2 is coimmunoprecipitated from either cell line. Combined, these results substantiate the existence of distinct ZO-1/ZO-2 and ZO-1/ZO-3 complexes. They further indicate that the level of ZO-1/ZO-3 can be increased at the expense of ZO-1/ZO-2 by the overexpression of ZO-3.

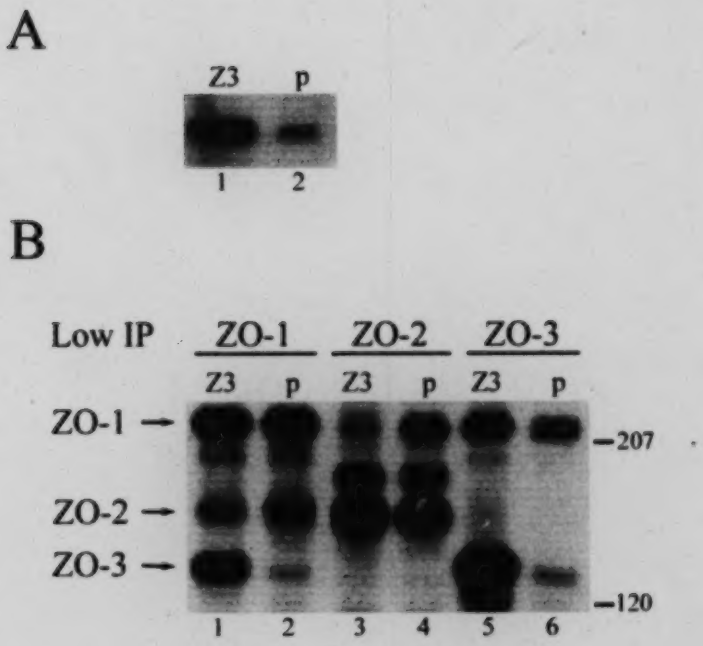



**Figure 2.6. Overexpression of ZO-3 in MDCK cells results in an increase in the ZO-1/ZO-3 complex and a decrease in the ZO-1/ZO-2 species.** (A) ZO-3 was immunoprecipitated from MDCK cells transfected with a full length ZO-3 construct (MDCK/Z3, lane 1) and from parental MDCK cells (lane 2) and immunoblotted with anti-ZO-3. Significantly more ZO-3 is present in the MDCK/Z3 cells. (B) Low stringency immunoprecipitations (Low IP) with anti-ZO-1, anti-ZO-2 and anti-ZO-3 from equivalent protein extracts of metabolically labeled MDCK/Z3 (lanes 1, 3, 5) and parental MDCK cells (lanes 2, 4, 6). The amount of ZO-2 coimmunoprecipitated with anti-ZO-1 is reduced in MDCK/Z3 cells (lane 1) compared to parental cells (lane 2). In the anti-ZO-2 immunoprecipitations, less ZO-1 is coimmunoprecipitated from MDCK/Z3 cells (lane 3) than from parental MDCK cells (lane 4). No ZO-3 is coimmunoprecipitated with the anti-ZO-2 antibody. In the anti-ZO-3 immunoprecipitations, more ZO-1 is coimmunoprecipitated in MDCK/Z3 cells (lane 5) than parental cells (lane 6), and no ZO-2 is coimmunoprecipitated. Molecular mass markers at right (kD).

## 2.4 Discussion

While considerable progress has been made in the identification of tight junction-associated proteins (refer to Table 1.1), the next level of understanding in tight junction biology will be gained by determining exactly how these proteins interact and how those interactions relate to tight junction physiology. In this study, we have explored the interactions of various tight junction proteins and the linkage of those proteins with the actin cytoskeleton. We endeavored to provide direct binding evidence, with the belief that such data will permit more accurate testing of the molecular mechanisms underlying function. By performing binding experiments with purified recombinant proteins *in vitro* we have identified novel binding interactions, and confirmed interactions that were previously suggested by indirect evidence.

### *2.4.1 Linking the Actin Cytoskeleton to the Tight Junction*

The results of our study indicate that there are other mechanisms for linking actin to the tight junction besides ZO-1, since ZO-2, ZO-3, and occludin can all directly interact with F-actin *in vitro*. More detailed characterization of this actin- tight junction protein interaction using actin cosedimentation experiments with sheared actin filaments revealed that ZO-2, ZO-3, and occludin do not bind F-actin ends and are likely side-binding proteins. Furthermore, based on low speed sedimentation assay results, it is unlikely that any of these three proteins have any cross-linking activity. This would indicate that ZO-2, ZO-3, and occludin act to tether actin filaments at the junctional membrane, and that different proteins may be involved in forming bundled actin networks. The *in vitro* actin cosedimentation data is further substantiated by the finding that ZO-2, ZO-3, and occludin colocalize with cytochalasin D-disrupted actin aggregates at cell borders *in vivo*. Since

this study was published, it has also been shown that cingulin can bind directly to F-actin in vitro (D'Atri and Citi, 2001). Together, these results indicate that actin has multiple potential binding partners at the tight junction. Whether all of these interactions occur simultaneously or shift depending on the physiological state of the tight junction remains to be determined.

The observed interactions between F-actin and ZO-1, ZO-2, and ZO-3 may be due to the homology and conserved domains present among the three ZO proteins. The data of Fanning *et al.*. (Fanning *et al.*, 1998) indicates the putative actin-binding domain of ZO-1 resides COOH-terminal to the GUK domain, an area that encompasses the proline-rich region. A particularly novel finding is that the transmembrane protein occludin can interact with F-actin either directly or indirectly via a linkage with ZO-1, ZO-2, or ZO-3. Given that we know occludin plays a role in modulating tight junction permeability (Balda *et al.*, 1996; McCarthy *et al.*, 1996; Chen *et al.*, 1997; Wong and Gumbiner, 1997), multiple linkage mechanisms for joining occludin to the actin cytoskeleton may provide the versatility required for differential regulation of permeability. Recently, Kojima *et al.*. (Kojima *et al.*, 1999) have also addressed the question of tight junction-cytoskeleton attachment. In rat hepatocytes, treatment with the actin depolymerizing drug mycalolide B caused disappearance of both the circumferential actin filaments and occludin, while intact tight junction strands remained, leading them to hypothesize that occludin, but not other transmembrane proteins play a role in the linkage between the actin cytoskeleton and tight junction.

*2.4.2. Direct Binding of ZO Proteins to Occludin*

Our finding that ZO-2 binds to the COOH-terminal 147 amino acids of occludin confirms and extends the result of Itoh *et al.*.(Itoh *et al.*, 1999a). Based on these results and previous work (Furuse *et al.*, 1997; Fanning *et al.*, 1998; Haskins *et al.*, 1998), we now have evidence that this region of occludin is capable of binding all three ZO proteins (ZO-1, ZO-2, and ZO-3). Differential binding of the ZO proteins to occludin may indicate functional redundancy, or may be a means of tight junction regulation. Since this study was published, different groups have identified other binding interactions involving transmembrane proteins and ZO-1, ZO-2, or ZO-3. The claudins also bind to all three ZO proteins *in vitro* (Itoh *et al.*, 1999b), and JAM binds directly to ZO-1 (Bazzoni *et al.*, 2000).

*2.4.3. Nature of the Binding Interactions Between ZO-1, ZO-2, and ZO-3*

Based on coimmunoprecipitation experiments (Balda *et al.*, 1993; Jesaitis *et al.*, 1994; Haskins *et al.*, 1998) and the fact that ZO-2 and ZO-3 do not interact directly (Haskins *et al.*, 1998) while ZO-1 binds to both ZO-3 (Haskins *et al.*, 1998) and ZO-2 (Figure 2.4), we originally hypothesized that ZO-1 may act as a linker mediating the association of ZO-1, ZO-2, and ZO-3 in a three-member complex. However, existing data could also be explained by the presence of two independent complexes of ZO-1/ZO-2 and ZO-1/ZO-3. We sought to resolve this issue by performing a series of low stringency coimmunoprecipitation experiments using antibodies to all three ZO proteins. These experiments provide evidence that ZO-1/ZO-2 and ZO-1/ZO-3 exist as independent complexes (Figures 2.5, and 2.6). If a trimeric ZO-1/ZO-2/ZO-3 complex exists, it is either not a predominant species, or it may be an undetectable transient phenomenon.

Further confirmation of two distinct complexes is provided by the finding that a shift in the normal balance of these two complexes can be induced in MDCK cells overexpressing ZO-3. One interpretation is that ZO-1 has a higher affinity for and will preferentially bind to ZO-3 rather than ZO-2 if ZO-3 levels are not limiting in the cell. A second interpretation is that in the presence of saturating levels of ZO-3, ZO-3 competes with ZO-2 for binding to ZO-1.

### *2.4.4. Conclusions*

In summary, the experiments outlined here reveal novel binding interactions among tight junction-associated proteins. A schematic representation of the binding interactions among tight junction proteins is shown in Figure 2.7. The model shown here is based on what was known at the time and is updated in the final chapter of this thesis (Figure 5.1).

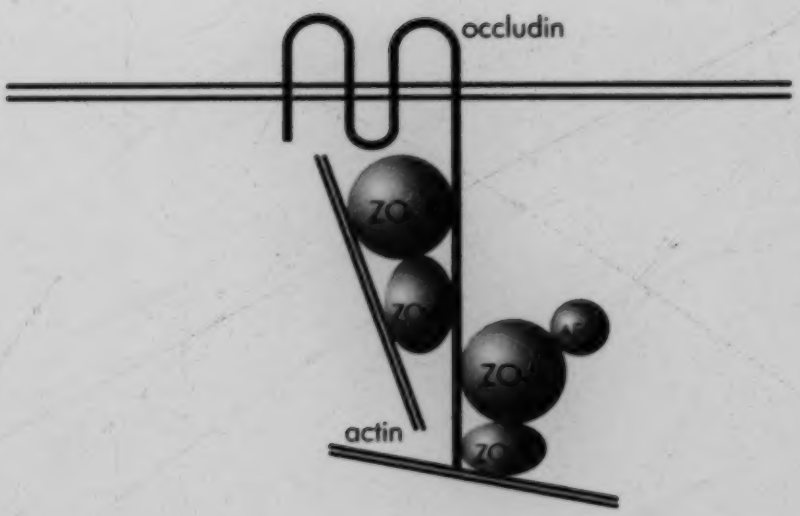



**Figure 2.7. Schematic diagram showing known tight junction protein interactions.** The results described in this chapter demonstrate that ZO-2 binds directly to ZO-1 and occludin, and ZO-2, ZO-3, and occludin can directly associate with the sides of actin filaments. Previously published results indicate that ZO-1 binds F-actin, although direct binding has not been demonstrated. Additionally, our evidence supports the existence of two independent complexes comprising of ZO-1/ZO-2 and ZO-1/ZO-3, rather than a 3-member ZO-1/ZO-2/ZO-3 complex. The binding interactions among other tight junction proteins and whether all of these interactions occur simultaneously remains to be determined.

Figure 2.7 provides a conceptual framework for the study of tight junction physiology, although much information is yet to be obtained. For example, it will be of interest to determine if all of these possible linkages exist at the tight junction simultaneously, or if there are preferential sub-groupings related to the physiological state of the tight junction. However, our findings reveal an intricacy that may be indicative of complex regulatory mechanisms, and/or functional redundancy.

## 2.5. Acknowledgements

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# CHAPTER 3

## Exogenous Expression of the Amino-terminal Half of the Tight Junction Protein ZO-3 Perturbs Junctional Complex Assembly [2]

[2] A version of this chapter has been published. Wittchen ES, Haskins J, Stevenson BR. 2000. Exogenous expression of the amino-terminal half of the tight junction protein ZO-3 perturbs junctional complex assembly. Reproduced from **The Journal of Cell Biology, 2000, 151(4):825-36** by copyright permission from The Rockefeller University Press.

J. Haskins contributed to Figures 3.2 - 3.10 (part A), and 3.11 (part D). B.R. Stevenson contributed to Figure 3.4.

## 3.1 Introduction

While the list of tight junction proteins has grown rapidly in recent years, the understanding of the functions of these proteins, particularly those peripherally associated with the tight junction membrane, is minimal. Determining how junctional proteins interact is a first step towards deciphering tight junction physiology at the molecular level.

### *3.1.1. Binding Interactions of Tight Junction Proteins*

The MAGUK proteins ZO-2 and ZO-3 have been shown to interact directly with ZO-1 (Haskins *et al.*, 1998; Wittchen *et al.*, 1999) but not with each other (Haskins *et al.*, 1998). In contrast to the ZO-1/ZO-2/ZO-3 heterotrimeric complex that was originally proposed, ZO-1/ZO-2 and ZO-1/ZO-3 likely exist as two distinct heterodimers *in vivo* (Wittchen *et al.*, 1999; Chapter 2, this thesis). All three ZO proteins interact directly with the carboxy-terminal 150 amino acids of occludin (Furuse *et al.*, 1994; Haskins *et al.*, 1998; Wittchen *et al.*, 1999; Chapter 2) and claudin 1–8 (Itoh *et al.*, 1999a). ZO-1, ZO-2, and ZO-3 also bind actin filaments (Itoh *et al.*, 1997; Fanning *et al.*, 1998; Wittchen *et al.*, 1999; Chapter 2), providing multiple linkage sites to the actin cytoskeleton. Furthermore, ZO-1, ZO-2, and ZO-3 all interact with another tight junction plaque protein, cingulin (Cordenonsi *et al.*, 1999), and ZO-1 can associate with the Ras-effector molecule AF-6 (Yamamoto *et al.*, 1997) and the integral membrane protein JAM (Bazzoni *et al.*, 2000).

The specific domains responsible for the various binding interactions of ZO-1, ZO-2, and ZO-3 have, in part, been defined. ZO-1 and ZO-2 form a complex through reciprocal interactions between their PDZ2 domains (Fanning *et al.*, 1998; Itoh *et al.*,

1999b), and ZO-3 likely interacts with ZO-1 in the same manner (Itoh *et al.*, 1999a). ZO-1 interaction with occludin likely occurs through the GUK domain, and actin binding via a region that encompasses the proline-rich domain of ZO-1 (Fanning *et al.*, 1998). ZO-1, ZO-2, and ZO-3 binding to the carboxy-terminal YV sequence of claudin 1–8 requires the first PDZ domain of each protein (Itoh *et al.*, 1999a). No further information on individual domain binding characteristics or overall protein function(s) is available for ZO-3.

*3.1.2. Junctional Complex Assembly*

The current model for junctional complex assembly involves the coordinated assembly of the adherens junction and the tight junction. This model proposes that the first step in *de novo* junction formation involves E-cadherin-mediated cell adhesion (Gumbiner *et al.*, 1988), followed by ZO-1 recruitment to the lateral surface of the cell via a transient interaction with the α/β-catenin complex (Rajasekaran *et al.*, 1996). Indeed, it was subsequently found that ZO-1 binds α-catenin *in vitro* (Itoh *et al.*, 1997), providing the means by which ZO-1 is associated with the catenin complex during this early stage in junction formation. As epithelial polarization proceeds, ZO-1 is sorted out of the adherens junction and forms part of a separate tight junction complex (Ando-Akatsuka *et al.*, 1999). *In vitro* binding experiments have shown that ZO-2 can also bind α-catenin (Itoh *et al.*, 1999b), indicating the possibility that ZO-2 may follow a similar path during initial stages of junctional complex assembly, either directly via interaction with α-catenin or indirectly via its association with ZO-1 (Wittchen *et al.*, 1999; Chapter 2, this thesis). Recent data suggest that this hierarchy of junctional complex assembly is not absolute. Troxell *et al.* (2000) have shown that tight junction assembly was

extensive in MDCK cells overexpressing a mutant E-cadherin protein lacking the extracellular domain required for cell-cell adhesion, suggesting the possibility that tight junctions can assemble in the absence of an initial E-cadherin-mediated cell adhesion event. However, a caveat to this result came from an earlier paper by the same group, where expressing the same mutant E-cadherin protein at lower levels resulted in faster disassembly of junctions and delayed reassembly during a calcium switch experiment (Troxell *et al.*, 1999). This suggests that other mechanisms of junctional assembly can eventually take over in the absence of E-cadherin-mediated signals.

### *3.1.3. Chapter Summary*

In this chapter, the functional characteristics of the tight junction protein ZO-3 were explored through exogenous expression of mutant protein constructs in MDCK cells. Expression of the amino-terminal, PDZ domain-containing half of the molecule (NZO-3) delayed the assembly of both tight junctions and adherens junctions induced by calcium switch treatment or brief exposure to the actin-disrupting drug cytochalasin D. The tight junction components ZO-1, ZO-2, endogenous ZO-3, and occludin were mislocalized during the early stages of tight junction assembly. Interestingly, we observed that the adherens junction proteins E-cadherin and β-catenin also exhibited delayed recruitment to the cell membrane, and NZO-3 expression had striking effects on actin cytoskeleton dynamics during junctional assembly. NZO-3 expression did not alter expression levels of ZO-1, ZO-2, endogenous ZO-3, occludin, or E-cadherin; however, the amount of Triton X-100–soluble, signaling-active β-catenin was increased in NZO-3-expressing cells during junction assembly. *In vitro* binding experiments showed that ZO-1 and actin preferentially bind to NZO-3, whereas both NZO-3 and the carboxy-terminal

half of the molecule (CZO-3) contain binding sites for occludin and cingulin. These studies provide the first functional information on ZO-3 and help to define how the protein constituents of the junctional complex may interact and assemble in epithelial cells.

## 3.2 Experimental Procedures

### *3.2.1. Plasmid Construction and MDCK Transfections*

The ZO-3 constructs used in this study are shown in Figure 3.1. A vesicular stomatitis virus glycoprotein (VSVG) epitope-tagged version of full-length ZO-3 (FLZO-3) in the expression vector pBK-CMV used for transfection of MDCK cells has been described (Haskins *et al.*, 1998). The stable cell line FLZO-3/MDCK was generated by transfection using Lipofectin (GIBCO BRL) and G418 selection as described (Haskins *et al.*, 1998). A construct composed of the amino-terminal half of ZO-3 (NZO-3) contains the three PDZ domains, arginine-rich region, and proline-rich domain (amino acids 1–454). For stable transfection in MDCK cells, this construct was subcloned into pBK-CMV with a VSVG epitope tag inserted at the carboxy terminus. NZO-3 was also subcloned into the pFast-Bac HT vector series of the Baculovirus eukaryotic expression system (GIBCO BRL) to generate a 6-histidine–tagged protein used for *in vitro* binding experiments. A construct composed of the entire carboxy-terminal half of ZO-3 (CZO-3) containing the SH3 and GUK domains, followed by the acidic region (amino acids 455–899) was subcloned into pBK-CMV with a VSVG-epitope tag at the carboxy terminus and stably transfected into MDCK cells. CZO-3 was also inserted into the pFastBac HT vector series to generate a 6-histidine–tagged recombinant protein for binding analyses.

For each stable cell transfection, at least two clones were selected and analyzed for expression of constructs by immunofluorescence and immunoblotting.

*3.2.2. Calcium Switch and Cytochalasin D Treatment*

For transepithelial resistance (TER) measurements and immunohistochemistry, untransfected or transfected MDCK cells grown as previously described (Howarth *et al.*, 1992) were plated on Transwell polycarbonate membrane filter inserts (0.4 μm pore size; Costar Corp.) coated with rat tail collagen at a cell density of $3 \times 10^5$ cells/cm$^2$. Cells were fed every day. Calcium switch experiments were performed with minor modifications of previously published methods (Cereijido *et al.*, 1978; Gumbiner and Simons, 1986). In brief, cells were trypsinized and replated on filters in DME containing 1.8 mM calcium for 2–3 h to allow attachment. Filters were then rinsed 2 x with PBS without added calcium (PBS$^-$) before switching to low calcium (5 μM) DMEM overnight (~15 h) to allow disassembly of the junctional complex. Cells were then switched back to 1.8 mM calcium-containing media at $t$=0, and junctional reassembly was followed for various times. Control cells used for determination of steady-state levels of NZO-3 were trypsinized, replated in normal calcium media, and incubated for~65 h.

Cytochalasin D (cD) (4μg/ml) in Hepes-buffered saline plus glucose (HBSG; 10 mM Hepes, pH 7.4, 5.4 mM KCl, 137 mM NaCl, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose), was added to filter-grown monolayers for 60 min and then was washed out by rinsing filters 3 x with HBSG. Cells were then incubated in HBSG without cD for another 2 h.

*3.2.3. Transepithelial Resistance Measurements*

TER was measured with a Millicell-ERS apparatus (Millipore) in HBSG as previously described (Stevenson and Begg, 1994). In the results depicted in Figure 3.4 (part A and B), duplicate filters from each cell line were measured for each time point, and the experiment was performed at least twice. Data from at least two experiments ($n$=4 filters) are presented as mean ± SEM. Data from single clones of parental, NZO-3/MDCK, CZO-3/MDCK, and FLZO-3/MDCK cells are shown. Two additional, independently selected NZO-3/MDCK cell lines were tested in both the calcium switch and cD experiments and showed results essentially identical to the first NZO-3/MDCK cell line (data not shown). For calcium switch experiments (see Figure 3.4, part A), TER is plotted in ohm/cm$^2$. For cD experiments (see Figure 3.4, part B), TER is expressed as a percentage normalized to $t$=0. The steady-state TER of all cell lines examined was 50–60 ohm/cm$^2$.

*3.2.4. Antibodies*

The following antibodies were used: rat anti–ZO-1 mAb R40.76 (Anderson *et al.*, 1988), rabbit anti–ZO-2 polyclonal Ab R9989 (Jesaitis *et al.*, 1994), rabbit anti-VSVG (a gift from Dr. Carolyn Machamer, Johns Hopkins University, Baltimore, MD), rabbit anti-occludin (Wong and Gumbiner, 1997; a gift from Drs. Alpha Yap and Barry Gumbiner, Memorial Sloan-Kettering Cancer Center, New York, NY), mouse anti–E-cadherin mAb 3G8, (a gift from Dr. Warren Gallin, University of Alberta, Edmonton, Canada), and rabbit anti-β-catenin (Sigma-Aldrich; No. C2206). For detection of ZO-3 constructs, the following antibodies were used: guinea pig anti–ZO-3 (Haskins *et al.*, 1998) and mouse anti–ZO-3 mAB3260 (Chemicon International, Inc.), which both recognize an epitope in

the N-terminus of ZO-3 and were used to detect full-length ZO-3 and NZO-3; rabbit anti–ZO-3 polyclonal AB3220 (Chemicon International, Inc.) recognizes an epitope in the extreme carboxy terminus of ZO-3 and was used to detect CZO-3. HRP-conjugated goat anti–rabbit and HRP-conjugated goat anti–mouse (Bio-Rad Laboratories), HRP-conjugated goat anti–guinea pig and HRP-conjugated anti–rat (Jackson ImmunoResearch Laboratories) were used as secondary antibodies for immunoblots. Rhodamine-conjugated donkey anti–rabbit, rhodamine-conjugated donkey anti–guinea pig, rhodamine-conjugated goat anti–rat, rhodamine-conjugated goat anti–mouse, FITC-conjugated goat anti–rat, and Texas red-conjugated goat anti–mouse (Jackson ImmunoResearch Laboratories) were used as secondary antibodies for indirect immunofluorescence.

*3.2.5. Immunohistochemistry*

For immunofluorescence staining with anti-VSVG, cells grown on collagen-coated coverslips were fixed with 5% acetic acid in ethanol for 5 min at -20°C and permeabilized with 0.2% Triton X-100 (TX-100) in TBS plus 1.8 mM calcium ($TBS^+$) for 4 min at room temperature. For all other staining, cells grown on filters were fixed with 2.5% paraformaldehyde in TBS and permeabilized with 0.2% TX-100. Rhodamine-conjugated phalloidin (Sigma-Aldrich) was used to stain F-actin. Coverslips and filters were viewed on a Zeiss Axioskop (Carl Zeiss, Inc.).

*3.2.6. Protein Expression Analysis and TX-100 Extraction*

*Expression Levels During Assembly:* Parental or NZO-3/MDCK cells grown on 35-mm tissue culture dishes were subjected to a calcium switch, and total lysates were made from cells 6, 12, 24, and 48 h after the re-addition of calcium. The steady-state

level of NZO-3 was assessed on control cells not subjected to calcium switch that were maintained in normal calcium media for ~65 h. Monolayers were rinsed 2 x with ice-cold $TBS^+$ and scraped up into 1 ml ice-cold $TBS^+$ plus protease inhibitor cocktail (1 μg/ml aprotinin, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM Pefabloc SC; Boehringer). Scraped cells were then harvested by centrifugation at 6,500 rpm for 5 min at 4°C. Cell pellets were resuspended in hot SDS lysis buffer (10 mM Tris, pH 7.8, 1% SDS) plus protease inhibitors and boiled for 5 min, shearing with a narrow bore pipette tip. Protein concentration was determined by BCA protein assay (Pierce Chemical Co.) and 20 μg total protein was loaded per lane on SDS-PAGE. For immunoblots, gels were electrophoretically transferred to nitrocellulose, immunoblotted with corresponding antibodies, and proteins detected via ECL (Amersham Pharmacia Biotech).

*Steady-state Construct Expression Levels:* Steady-state construct expression levels of two independent NZO-3/MDCK cell lines (D5 and B6) and one CZO-3/MDCK cell line were determined as above, except that cells were not subjected to a calcium switch (see Figure 3.2).

*CSK Extraction of Soluble and Insoluble β-catenin:* For separation of TX-100-soluble and –insoluble β-catenin, parental and NZO-3/MDCK cells subjected to a calcium switch were extracted with CSK buffer (10 mM Pipes, pH 6.8, 50 mM NaCl, 300 mM sucrose, 0.5% TX-100 plus 0.1 mg/ml DNAse, 0.1 mg RNAse, and protease inhibitor cocktail; Troxell *et al.*, 2000). In brief, cell monolayers grown on 35-mm dishes were rinsed 2 x with ice-cold $TBS^+$ and then extracted for 10 min on ice with 1.5 ml CSK buffer. Insoluble material was scraped from the dish, and the extracts were centrifuged

10 min at 13,000 rpm at 4°C. Supernatants contain the detergent-soluble pool of β-catenin. Pellets containing detergent-insoluble material were resuspended in 1.5 ml hot SDS lysis buffer and boiled for 10 min. Protein concentration in each fraction was determined by BCA protein assay, and samples were loaded for SDS-PAGE and immunoblot analysis as above.

*3.2.7. Recombinant Protein Expression and In Vitro Binding Studies*

Recombinant 6-histidine–tagged NZO-3 and CZO-3 were expressed in Sf9 insect cells using a Baculovirus eukaryotic expression system (GIBCO-BRL) and purified according to previously published methods (Haskins *et al.*, 1998; Wittchen *et al.*, 1999). Empty expression vector encoding the 6-histidine tag plus 36 nonspecific amino acids served as the negative control. Equal amounts of purified NZO-3 and CZO-3 were used in the binding experiments. [$^{35}$S]-methionine-labeled *in vitro*–transcribed/translated human ZO-1 was prepared as described (Haskins *et al.*, 1998; Wittchen *et al.*, 1999). Expression of GST-occludin encoding the carboxy-terminal 150 amino acids of the cytoplasmic tail of chicken occludin (Furuse *et al.*, 1993) was described previously (Haskins *et al.*, 1998; Wittchen *et al.*, 1999). GST–N-cingulin cDNA encoding the N-terminal head region (amino acids 1–378) of *Xenopus* cingulin in pGEX-4T1 (a gift from Dr. Sandra Citi, University of Geneva, Geneva, Switzerland) was expressed according to published methods (Cordenonsi *et al.*, 1999).

The binding of NZO-3 and CZO-3 to actin, ZO-1, and occludin was performed as previously described (Wittchen *et al.*, 1999). Cingulin binding experiments used a modification of the methods of Cordenonsi et al (1999). In brief, GST–N-cingulin or GST alone was immobilized on glutathione-Sepharose 4B beads, and equal amounts of

purified NZO-3 or CZO-3 were added. Binding buffer consisted of PBS plus 1% TX-100 and protease inhibitors. Binding was allowed to proceed overnight at 4°C, followed by washing with the same buffer, and eluting bound protein by boiling the beads in gel sample buffer. NZO-3 and CZO-3 binding was detected by immunoblot, and in the case of occludin and cingulin binding experiments, the amount of bound NZO-3 and CZO-3 was compared by Coomassie blue staining of the bound fraction.

## 3.3 Results

### *3.3.1. Localization of ZO-3 Constructs*

We first determined which region of ZO-3 encodes the information for targeting the protein to the tight junction. MDCK cells were stably transfected with the VSVG-tagged FLZO-3, NZO-3, and CZO-3 constructs (Figure 3.1). Expression of each construct was confirmed by western blot (Figure 3.2; Figure 2.6 in Chapter 2, this thesis). NZO-3/MDCK cell line D5 was chosen for use in the rest of the experiments shown in this chapter. Relative to endogenous ZO-3, the steady state expression level of the NZO-3 construct is only slightly lower (data not shown). Subcellular localization of the constructs was detected by indirect immunofluorescence using anti-VSVG antibodies and costaining for ZO-1 as a marker for the tight junction. FLZO-3 correctly targets to cell borders, colocalizing with the tight junction marker ZO-1 (data not shown). The NZO-3 construct also colocalizes with endogenous ZO-1 at cell borders (Figure 3.3); furthermore, its localization was also indistinguishable from endogenous ZO-1 as assayed by 0.2 μm Z-section confocal microscopy (data not shown). The nuclear staining visible

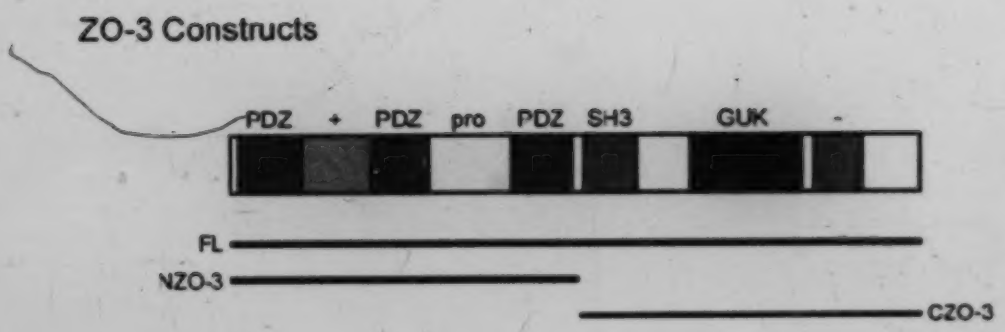



**Figure 3.1. Schematic diagram of the ZO-3 constructs used in this study.** Full-length ZO-3 (FLZO-3) contains three PDZ domains, an arginine-rich basic region (+), a proline-rich region (pro), an SH3 domain, a GUK domain, and an acidic region (-). NZO-3 comprises the amino-terminal half of ZO-3 and contains the three PDZ domains, basic region and proline-rich region. The CZO-3 construct corresponds to the carboxy-terminal half of the molecule and contains the SH3, GUK, and acidic domains. The constructs used for generating stably transfected MDCK cells have a VSVG epitope tag at their COOH-terminus.

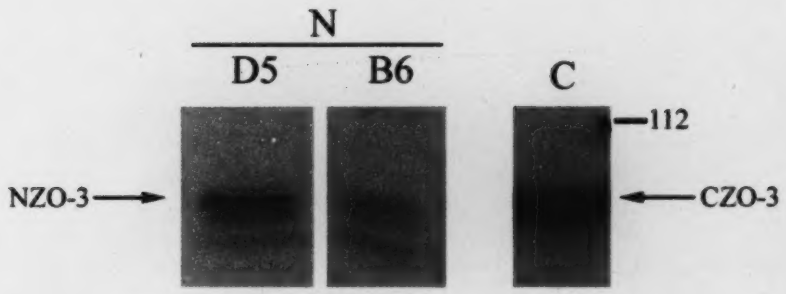



**Figure 3.2. Expression of ZO-3 constructs in MDCK cell lines.** Lanes 1 and 2: 20 μg of total lysate from NZO-3/MDCK cells lines D5 and B6 was blotted with anti-ZO-3 antibodies to detect the exogenously expressed NZO-3 construct. Note that the D5 cell line expresses NZO-3 at a higher level than the B6 cell line. Lane 3: 20 μg of total lysate from one of the CZO-3/MDCK cell line used in this study was blotted with anti-VSVG antibodies to detect the exogenously expressed CZO-3 construct. Since different antibodies were used to detect NZO-3 versus CZO-3, a comparison of expression levels of the two different constructs cannot be made. Molecular weight indicated at right (kD).

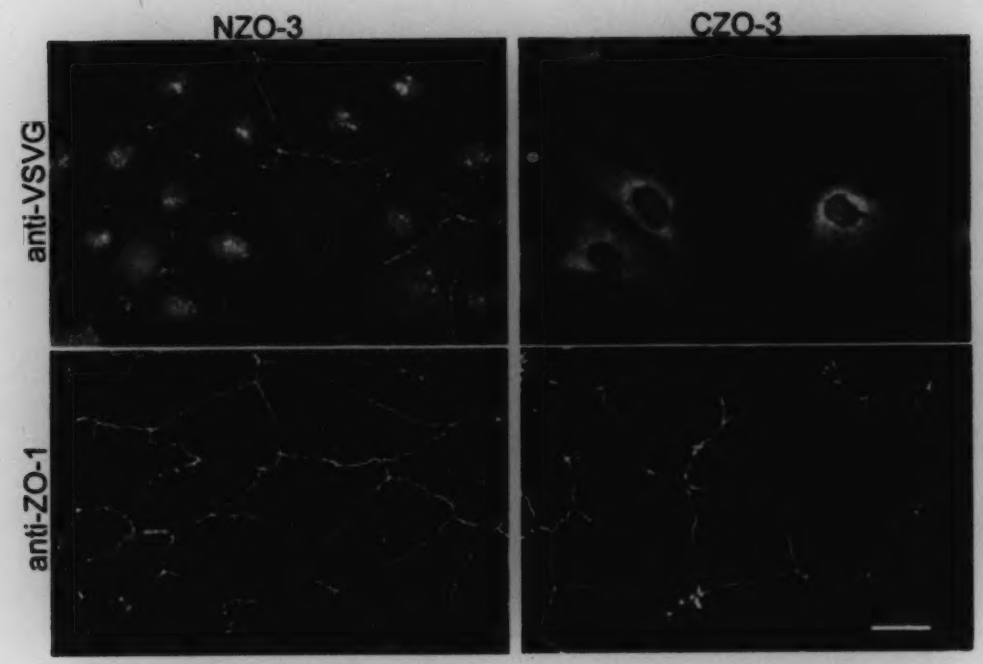



**Figure 3.3. The NZO-3 construct correctly targets to the tight junction; the CZO-3 construct does not.** MDCK cells were stably transfected with VSVG-tagged NZO-3 (NZO 3/MDCK) or CZO-3 (CZO-3/MDCK) constructs. Transfected cells were costained with anti–ZO-1 and anti- VSVG antibodies to mark the tight junction and localize the constructs by indirect immunofluorescence. NZO-3 precisely colocalizes with ZO- 1 at cell borders, with additional staining visible in the cytoplasm. CZO-3 is found mainly in the cytoplasm and is absent from cell borders. The nuclear staining in the NZO-3/MDCK cells stained with anti-VSVG is nonspecific (see Figure 3.6). Bar, 15 μm.

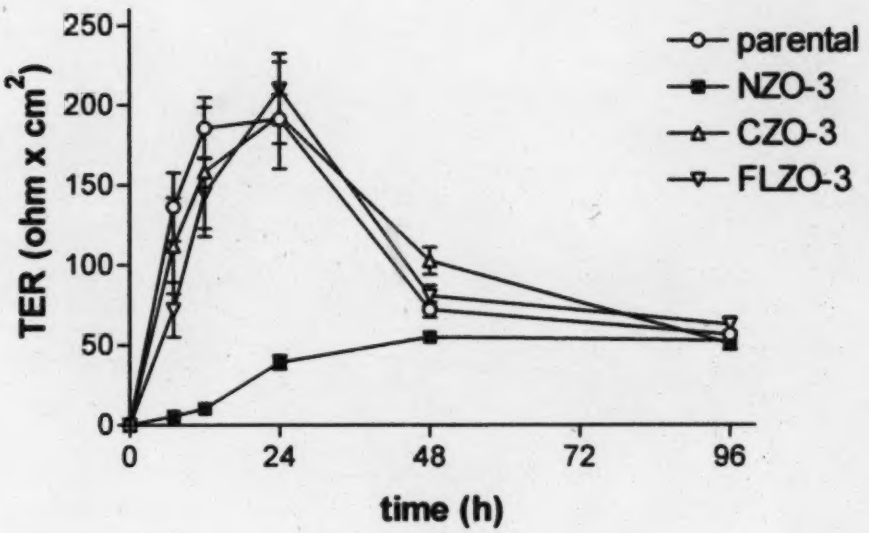
with the anti-VSVG staining is nonspecific as the same staining pattern is seen in untransfected parental MDCK cells (see Figure 3.6, part A). Figure 3.3 also shows that when expressed in MDCK cells, CZO-3 is absent from cell borders and is instead diffusely distributed in the cytoplasm. Combined, these data indicate that the information necessary for proper targeting of ZO-3 to the tight junction is contained within the N-terminal half of the protein.

*3.3.2. The Effect of Truncated ZO-3 Constructs on Tight Junction Physiology*

We next examined the effect of exogenous expression of these constructs on tight junction formation using two experimental paradigms. The first approach is based on the observation that adherens junctions and tight junctions are disrupted if epithelial cells are grown in medium containing subphysiological calcium concentration (5 μM). Subsequent restoration of physiological levels of calcium (1.8 mM) results in the synchronous *de novo* assembly of both junction types (Cereijido *et al.*, 1978; Gumbiner and Simons, 1986). Filter-grown monolayers of parental, NZO-3, CZO-3, or FLZO-3–expressing MDCK cells were grown overnight in low calcium media. Physiological concentration of calcium was added back at $t$=0, and tight junction assembly was monitored by measuring TER over a 48-h time period. As shown in Figure 3.4 (part A), parental MDCK cells undergo an initial rapid increase in TER to over 175 ohm/cm2 by 12 h. Resistance peaked at 12–24 h, before declining and leveling off to ~50 ohm/cm$^2$ by 96 h. Cell lines expressing the CZO-3 and FLZO-3 constructs followed a similar trend, plateauing at the same steady-state level as parental cells. In contrast, NZO-3-expressing cells exhibited delayed TER development, with no initial overshoot typical of the other

**Figure 3.4. Expression of NZO-3 delays tight junction formation.** (A) NZO-3 expression in MDCK cells subjected to a calcium switch delays reestablishment of TER. Filter-grown confluent monolayers of untransfected parental cells or MDCK cell lines stably expressing NZO-3, CZO-3, or FLZO-3 were incubated in calcium-free media for ~15 h to disrupt intercellular junctions and then switched to media containing 1.8 mM calcium at $t$ =0 to induce synchronous assembly of intercellular junctions. TER was measured over 96 h after calcium switch. CZO-3 and FLZO-3 expressing cell lines show TER recovery dynamics similar to parental cells. NZO-3–expressing cells display a delay in TER recovery, but reach the same TER as the other cell lines by 48–96 h. (B) Expression of NZO-3 delays TER recovery in MDCK cells after cD treatment. cD was added to filter grown confluent monolayers at $t$ =0 and then washed out after 60 min. TER measurements were taken every 30 min to monitor tight junction breakdown and reformation. TER for each cell line is expressed as percentages of the values at $t$=0. After 60 min of cD treatment, all cell lines exhibit a significant drop in TER. After cD washout, CZO-3/MDCK, FLZO-3/MDCK and parental cells recover TER to values greater than $t$=0, whereas NZO-3/MDCK cells do not fully recover TER for the duration of the experiment.

A


250
200
150
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TER (ohm x cm$^2$)
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time (h)
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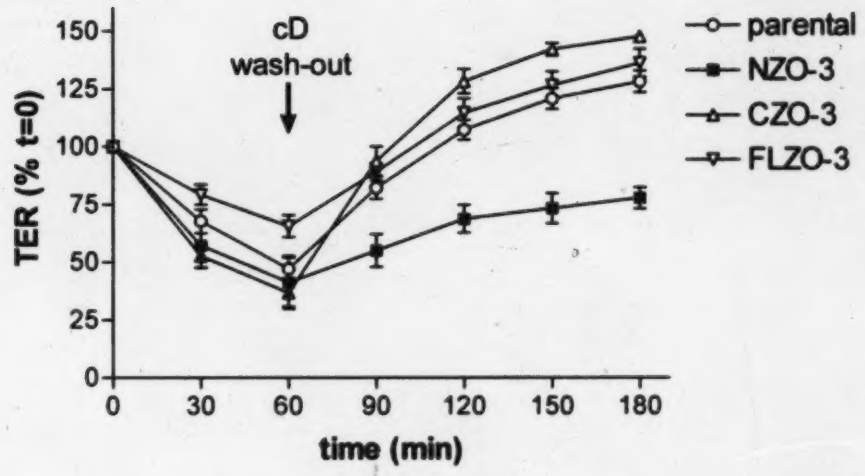
FLZO-3

B


150
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TER (% t=0)
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time (min)
parental
NZO-3
CZO-3
FLZO-3

cell lines. However, regardless of the delay, NZO-3-expressing cells reached a steady-state TER similar to that of the other cell lines by 48–96 h. A TER of ~50 ohms/cm$^2$ in NZO-3/MDCK cells at the 96 h time point corresponds to the normal level for untransfected MDCK IIm cells (50-70 ohms/cm$^2$; Stevenson *et al.*, 1988).

We corroborated the results obtained in the calcium switch experiments by determining the effect of ZO-3 construct expression on tight junction reformation in MDCK cells treated with the actin-disrupting drug cytochalasin D (cD). In cells treated with cD, the apical actin cytoskeletal ring associated with the junctional complex is disrupted into large punctate aggregates that colocalize with similarly disrupted tight junction proteins ZO-1, ZO-2, ZO-3, and occludin (Stevenson and Begg, 1994; Wittchen *et al.*, 1999). The cD-treated cells also show a sharp drop in TER, indicating a loss of functional tight junctions. It was shown that this effect is reversible, and upon washout of cD, tight junctions reassemble synchronously (Madara *et al.*, 1986; Stevenson and Begg, 1994). We treated our MDCK cell lines with cD for 60 min, and the drug was then washed out (Figure 3.4, part B). Upon cD treatment all cell lines exhibited an immediate and substantial decrease in TER. After cD washout, parental, FLZO-3–, and CZO-3–expressing cells recovered TER values equal to or greater than t=0 values within 1 h. However, NZO-3–expressing cells showed a delayed TER recovery from cD treatment and failed to reach $t$=0 levels for the duration of the experiment. This result is in agreement with the calcium switch experiment (Figure 3.4, part A) and demonstrates that MDCK cells expressing the NZO-3 construct display an impaired ability to assemble functional tight junctions.

### *3.3.3. Tight Junction Protein Localization During Junctional Complex Assembly*

The physiological data suggesting impaired tight junction-forming ability in NZO-3-expressing cells prompted us to assess the localization of proteins associated with both tight junctions and adherens junctions in the calcium switch experimental system. Parental and NZO-3/MDCK cells grown on filters were subjected to a calcium switch and protein localization was determined 6, 12, 24, and 48 h after re-addition of calcium. As shown in Figure 3.5 (part A), ZO-1 is localized at cell–cell contacts within 6 h after re-addition of calcium. In the 12–48-h time period, the parental cells develop the cobblestone uniformity of shape and size that is typical of an MDCK epithelial cell monolayer at steady-state. The appearance of ZO-1 at cellular contacts 6 h after re-addition of calcium correlates well with the rapid increase in TER observed in the parental cells (Figure 3.4, part A). In contrast, cells expressing NZO-3 showed a significant delay in ZO-1 recruitment to cell borders after re-addition of calcium (Figure 3.5, part A). At 6 h, only small, isolated clusters of cells showed ZO-1 staining at cell borders. Over time the areas of cell monolayer that show ZO-1 localization at cell-cell contacts increase in size. At 6, 12, and 24 h when ZO-1 is not yet fully localized to cell borders, ZO-1 also localizes to large intracellular aggregates and linear planes of contact between neighboring cells. Complete localization of ZO-1 at the junctional membrane in NZO-3-expressing cells did not occur until 48 h after re-addition of calcium. ZO-1 localization in both cell types at 48 h is indistinguishable from untreated, steady-state cells. Both parental and NZO-3/MDCK cells displayed no detectable differences in expression levels of ZO-1 (or any other junctional protein examined; see Figure 3.10 immunoblot) at 6, 12, 24, and 48 h. The delayed targeting of the tight junction protein

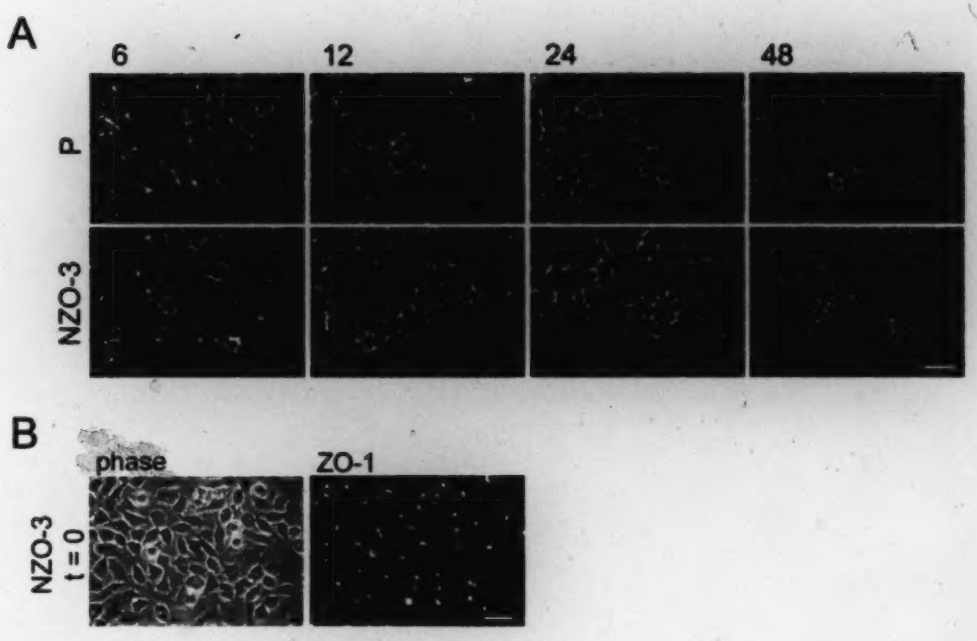



**Figure 3.5. NZO-3 expression alters the distribution of ZO-1 during the early stages of tight junction assembly.** (A) Parental MDCK (P) and NZO-3/MDCK (NZO- 3) cells were subjected to a calcium switch, and the localization of ZO-1 at 6, 12, 24, and 48 h after the re-addition of calcium was determined by immunofluorescence with anti–ZO-1 antibody. In parental cells, ZO-1 is completely localized to cell borders after 6 h in calcium-containing media. Over 48 h the cells become more tightly packed and uniform in size. In NZO-3 expressing cells, only scattered islands of cells showed ZO-1 localization at cell borders by 6 h. The number of cells with ZO-1 localized to cell–cell contact sites increases over time and is complete by 48 h after re-addition of calcium. (B) No NZO-3/MDCK cells show ZO-1 localization around their peripheries at $t$=0 in the calcium switch. Phase-contrast image shows that cells form a confluent monolayer at $t$=0 of calcium switch. Anti–ZO-1 immunofluorescence shows ZO-1 staining in large intracellular aggregates and in thick bars at sites where two cells make contact. Bars: (A and B) 30 μm.

ZO-1 in NZO-3/MDCK cells correlates with the delay in TER recovery after calcium switch (Figure 3.4, part A). The localization of the tight junction proteins ZO-2, endogenous ZO-3, and the integral membrane protein occludin were identical to that of ZO-1 in the NZO-3/MDCK cells (data not shown).

To rule out the possibility that the isolated islands of NZO-3/MDCK cells showing ZO-1 staining encircling cell peripheries at 6 h arose from cell clusters that did not disassemble their tight junctions during incubation in low calcium, and to demonstrate that the recruitment of ZO-1 to cell peripheries occurred *de novo*, we looked at ZO-1 localization in NZO-3/MDCK cells before the re-addition of calcium ($t$=0). As viewed by phase-contrast microscopy, cells plated at an identical density on glass coverslips show a confluent distribution at $t$=0, although the cells are somewhat retracted from each other due to the dissociation of adherens junctions and tight junctions in low calcium (Figure 3.5, part B). ZO-1 staining is completely disrupted, and is present only in large intracellular aggregates and thick, linear planes of contact between neighboring cells. No cells show ZO-1 staining encircling their peripheries at $t$=0. Thus, ZO-1 (and ZO-2, endogenous ZO-3, and occludin; data not shown) is recruited from an intracellular location to cell–cell contacts upon readdition of calcium, and this recruitment process is delayed in NZO-3-expressing MDCK cells.

The disrupted localization of tight junction proteins during junctional assembly after calcium switch in cells expressing NZO-3 indicates that this construct interferes with the formation of the complex of proteins found at the tight junction. We therefore examined the localization of the construct itself during this process. The anti-VSVG antibody only weakly recognizes its epitope on NZO-3 in immunofluorescence and was

not visible in filter-grown cells. Hence, NZO-3/MDCK cells grown on coverslips and subjected to a calcium switch were costained with the anti–ZO-1 and anti-VSVG (NZO-3) antibodies. Parental MDCK cells stained with anti-VSVG serve as a negative control and show that this antibody exhibits nonspecific nuclear staining in untransfected cells (Figure 3.6, part A). At the earliest time points (6,12, and 24 h), the NZO-3 construct appears at only those cell borders that also show ZO-1 localization. NZO-3/MDCK cells stained with anti-VSVG antibody also exhibit a faint, granular cytoplasmic staining above background. NZO-3 staining is only faintly visible at 48 h, likely due to the lower levels of construct expression at this time point (see Figure 3.10, immunoblot). In areas where NZO-3 is visible, it was colocalized with ZO-1 at all cell–cell contacts. Interestingly, NZO-3 was absent from the intracellular aggregates and linear planes of ZO-1 staining observed at early time points, and was only colocalized with ZO-1 at points of cell-cell contact formed by mature tight junctions (Figure 3.6, part B).

*3.3.4. F-actin Localization During Junctional Complex Assembly*

The actin cytoskeleton is an important structural and a functional element of the tight junction (Hirokawa and Tilney, 1982; Madara, 1987). Actin filaments have been shown to interact directly with multiple tight junction proteins (Itoh *et al.*, 1997; Fanning *et al.*, 1998; Wittchen *et al.*, 1999). Disruption of the actin cytoskeleton is associated with impaired barrier function of the tight junction (Madara *et al.*, 1986; Madara and Pappenheimer, 1987; Stevenson and Begg, 1994), whereas the fence function appears to be unaffected (Takakuwa *et al.*, 2000). Actin localization during tight junction assembly in the calcium switch system was studied by rhodamine-phalloidin staining of actin filaments (Figure 3.7). In parental MDCK cells, actin recruitment to a ring-like structure

**Figure 3.6. NZO-3 colocalizes with ZO-1 around cell borders during tight junction assembly after calcium switch**. (A) Parental MDCK (P) and MDCK/ NZO-3 (NZO-3) cells were subjected to a calcium switch and then costained with anti– ZO-1 and anti-VSVG (NZO- 3) antibodies at 6, 12, 24, and 48 h after the re-addition of calcium. Parental MDCK cells stained with anti-VSVG antibodies show nonspecific nuclear staining. In MDCK/ NZO-3 cells, NZO-3 is present at cell borders of small groups of cells where it colocalizes with ZO-1. These groups of cells showing NZO-3 and ZO-1 around their peripheries enlarge over time, with complete localization at cell borders by 48 h. Boxed areas are enlarged in B. (B) NZO-3 colocalizes with ZO-1 along extended cell peripheries, but not in intracellular aggregates or initial planes of cell– cell contact. Shown is a cell with mature tight junction staining (asterisk) where ZO-1 and NZO-3 colocalize. However, NZO-3 does not colocalize with ZO-1 at initial cell–cell contact planes (arrow) or in large intracellular aggregates (arrowheads). Bars: (A) 30 μm; (B) 10 μm.

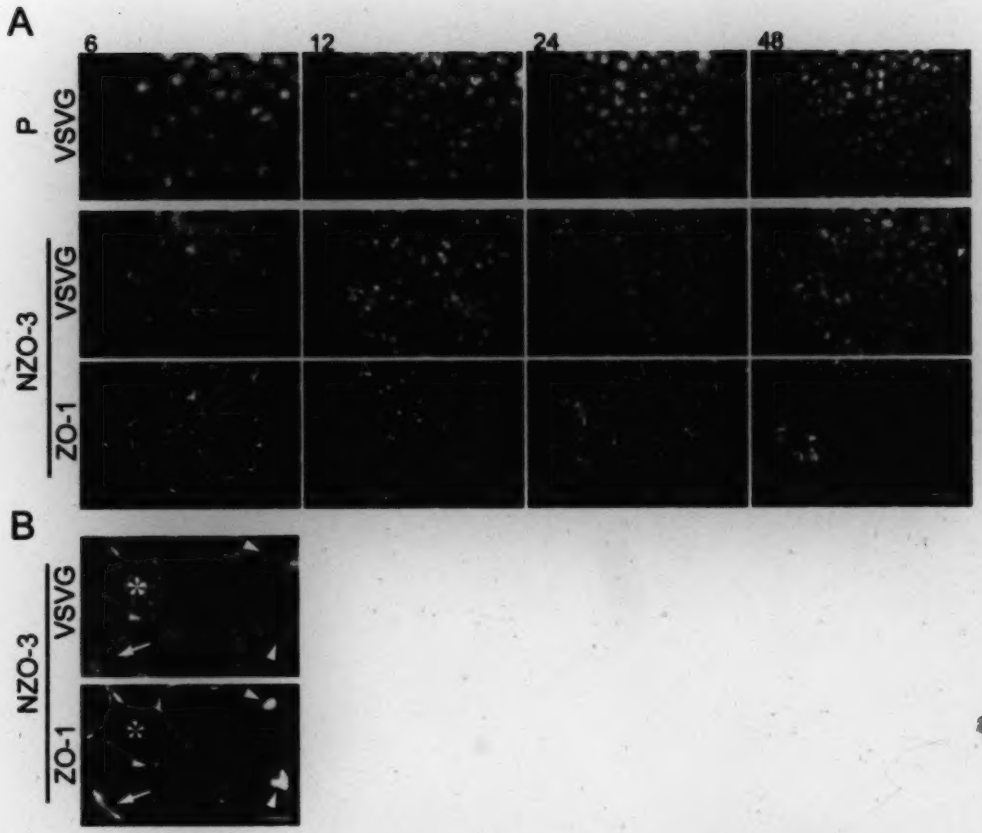



**Figure 3.7. NZO-3 expression disrupts actin filament organization during early stages of junction assembly.** (A) Parental MDCK (P) and NZO-3/MDCK (NZO-3) cells were subjected to a calcium switch. Actin filaments were visualized with rhodamine-phalloidin at 6, 12, 24, and 48 h after the readdition of calcium. In parental cells, actin is concentrated in an apical circumferential ring at the junctional complex in all cells after 6 h in normal calcium. NZO-3/MDCK cells show actin staining present in large aggregates at early time points (6, 12, and 24 h), with small clusters of cells containing a prominent apical actin ring, similar to the observations with ZO-1 and NZO-3 (Figs. 4 and 5). Actin is not localized to the apical circumferential ring in all cells until 48 h after calcium re-addition. Boxed areas of 12 h images are enlarged in B. (B) In NZO-3/MDCK (NZO-3) cells at 12 h, F-actin aggregates (arrow) are seen in cells which have not fully developed an apical actin ring. At the same time point, parental cells (P) have conspicuous apical actin rings and intracellular aggregates are absent. Bars: (A) 30 μm; (B) 15 μm.

at the apical aspect of the lateral cell surface occurs rapidly after re-addition of calcium; as early as 6 h all parental cells show a circumferential actin ring. This ring thickens and condenses as the cells become more tightly packed and uniform in size in the 12–48-h time period. However, exogenous expression of NZO-3 delays recruitment of actin to the apical ring in a manner similar to that observed with ZO-1. Development of an apical actin ring at points of cell–cell contact in NZO-3/MDCK cells occurred only in isolated patches of cells at 6 h, with the final localization not complete until 48 h. In addition, at the earlier time points (6, 12, and 24 h), actin also localized to large aggregates in cells which show underdeveloped actin rings. The delayed association of actin filaments with the junctional complex in NZO-3/MDCK cells is likely a factor in the delayed tight junction barrier formation in these cells.

*3.3.5 Adherens Junction Protein Localization During Junctional Assembly*

As the prevalent model of tight junction formation involves coordinated assembly with the adherens junction, we were interested to determine if E-cadherin was delayed in its movement to the junctional complex in NZO-3/MDCK cells in a calcium switch. In parental cells, E-cadherin appears at all cell–cell contacts 6 h after re-addition of calcium (Figure 3.8). This time course of E-cadherin recruitment to the adherens junction mirrors that of tight junction assembly and recruitment of ZO-1 to the tight junction (Figure 3.5, part A). Similar to ZO-1, E-cadherin localization during the calcium switch was also delayed in NZO-3–expressing cells (Figure 3.8). At 6 h, E-cadherin is localized to cell–cell contacts in small, isolated clusters of cells; these cell clusters also show ZO-1 localization to the junctional complex. An increasing number of cells show junctional E-cadherin staining at 12 and 24 h, but it is not until 48 h after calcium re-addition that E-

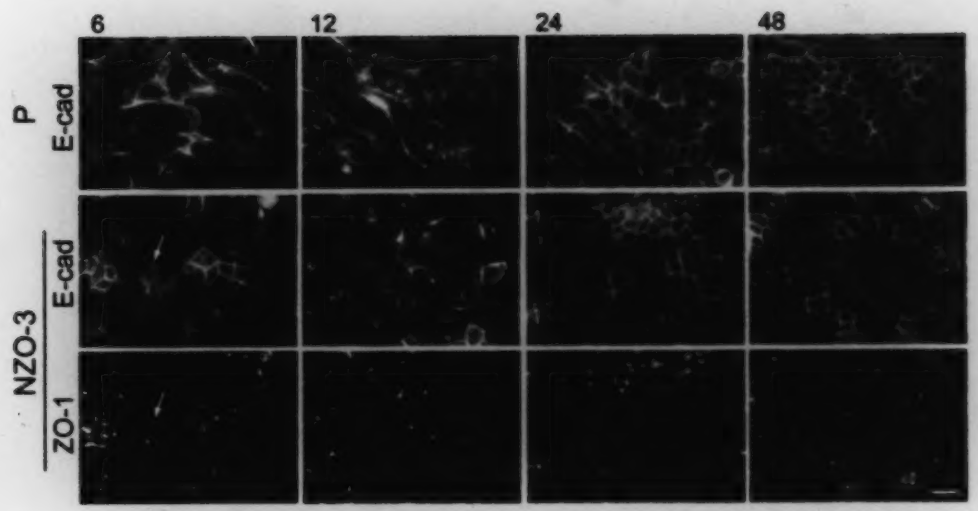



**Figure 3.8. NZO-3 expression delays recruitment of E-cadherin to cell–cell contacts.** Parental (P) and NZO-3/MDCK (NZO- 3) cells were subjected to a calcium switch, and E-cadherin and ZO-1 localization was monitored by costaining at 6, 12, 24, and 48 h after the re-addition of calcium. In parental cells, E-cadherin is localized to cell borders by 6 h. By 48 h, cell monolayers have an organized morphology. NZO-3–expressing cells costained with anti–ZO-1 and anti–E-cadherin antibodies show a delayed E-cadherin recruitment to cell borders. The clusters of cells that show E-cadherin localization at cell peripheries at early time points coincide with cells showing a similar distribution of ZO-1. Some cells (arrow) show E-cadherin at cell borders preceding ZO-1. E-cadherin staining at cell borders is complete by 24 h after the readdition of calcium, whereas ZO-1 staining at cell peripheries is not complete until 48 h. Bar, 30 μm.

cadherin is completely recruited to all cell borders in NZO-3/MDCK cells. In addition, we identified cells where junctional recruitment of E-cadherin precedes that of ZO-1. The disruption of E-cadherin localization in NZO-3/MDCK cells after calcium switch indicates that adherens junctions and tight junctions are both affected by exogenous expression of the NZO-3 construct. We corroborated our E-cadherin findings by assessing the localization of β-catenin, an adherens junction protein peripherally associated with the membrane (Figure 3.9). In parental cells, β-catenin appears at cell-cell contacts 6 h after calcium re-addition in a manner identical to the localization of E-cadherin after calcium switch (data not shown). However, in a distribution similar to E-cadherin and ZO-1, β-catenin recruitment to the lateral cell surface during junctional complex assembly was delayed in NZO-3/MDCK cells.

*3.3.6. Protein Biochemistry*

In addition to the altered localization of junctional complex proteins and disruption of the actin cytoskeleton, it is possible that the perturbation of junctional assembly in NZO-3-expressing cells is also due to changes in the expression levels of component proteins. Equivalent protein loadings of whole cell lysates harvested from parental and NZO-3/MDCK cells at 6, 12, 24, and 48 h after readdition of calcium were immunoblotted for the NZO-3 construct, endogenous ZO-3, ZO-1, ZO-2, occludin, and E-cadherin (Figure 3.10, part A). As expected, no NZO-3 was detected in untransfected parental cells. NZO-3 expression in NZO-3/MDCK cells increased in response to the calcium switch, with highest expression at 6 h. Construct expression then gradually decreased over time and returned to stable, steady-state levels. This decrease over time correlates well with recovery of TER and the normal localizations of junctional proteins

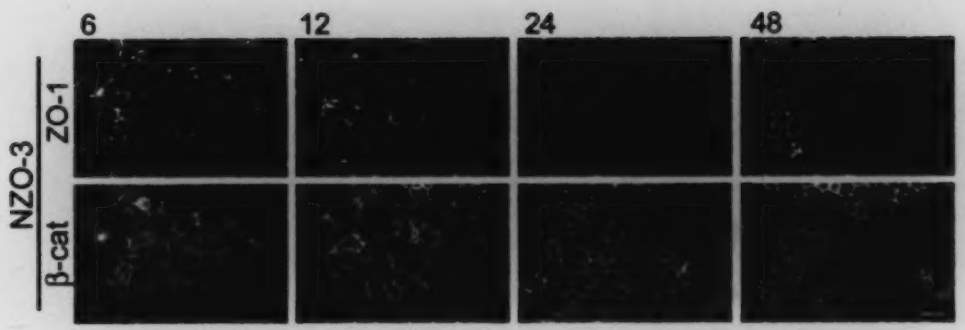
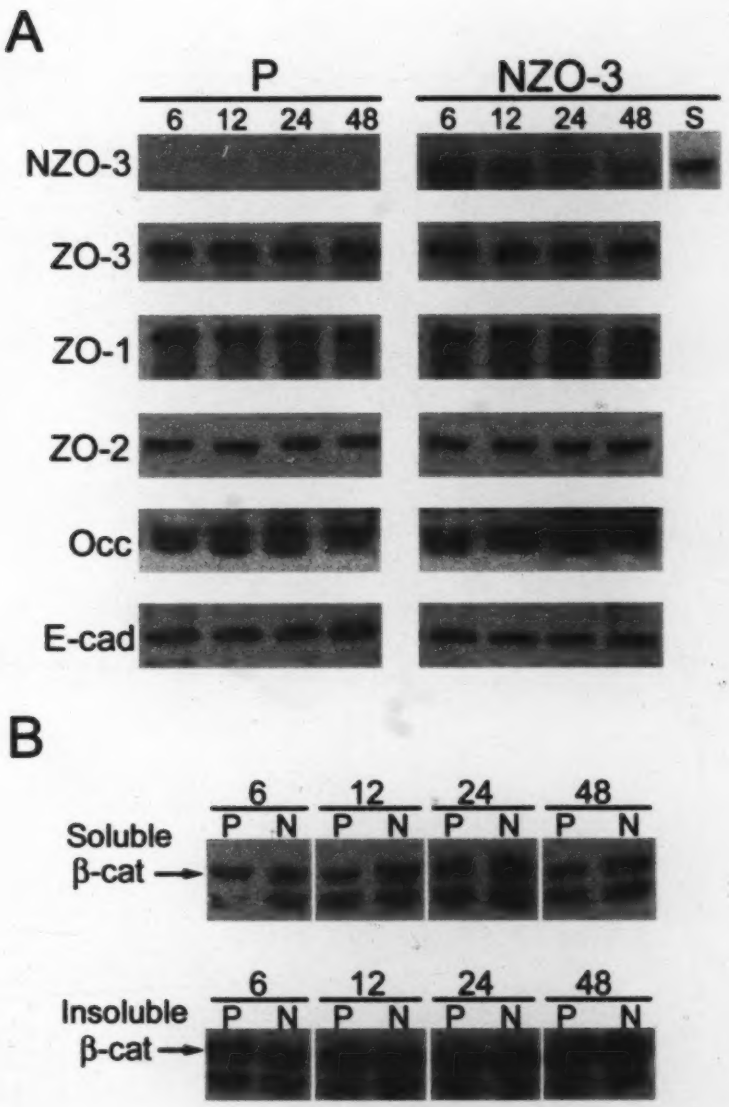



**Figure 3.9. NZO-3 expression delays recruitment of β-catenin to cell–cell contacts.** β-catenin and ZO-1 localization in NZO-3/MDCK cells was monitored at 6, 12, 24, and 48 h after the re-addition of calcium in a calcium switch experiment. NZO-3– expressing cells show a delayed β-catenin recruitment to intercellular junctions, which parallels the delay of E-cadherin recruitment (Fig. 7). The cells that show β-catenin localization at cell peripheries at early time points coincide with cells showing similar distribution of ZO-1, although many cells show β-catenin at cell borders preceding ZO-1. β-Catenin staining at cell borders is not complete until 24 h after the re-addition of calcium in NZO-3/MDCK cells. Bar, 30 μm.

**Figure 3.10. Expression levels of tight junction and adherens junction proteins during a calcium switch experiment.** (A) Equivalent protein loadings of whole cell lysates of parental (P) and NZO-3/MDCK (NZO-3) cells were analyzed by immunoblot at 6, 12, 24, and 48 h after the re-addition of calcium. The monoclonal antibody against ZO-3 was used to detect both the NZO-3 construct and endogenous ZO-3; the proteins were distinguished by their difference in molecular mass. NZO-3 is not detected in parental cells. NZO-3 expression in NZO-3/MDCK cells increases in response to the calcium switch and then over time returns to stable, steady-state levels (S). Endogenous ZO-3, ZO-1, ZO-2, occludin, and E-cadherin levels are unchanged during the course of the calcium switch experiment in both cell lines, and no significant differences in these protein levels are detected between parental and NZO-3/MDCK cells at any time points. Occludin shows a shift to a higher molecular weight species in both cell lines over time. (B) NZO-3/MDCK cells have more soluble β-catenin than parental cells. At indicated time points after a calcium switch, parental (P) and NZO-3/MDCK (N) cells were extracted with CSK buffer (see Experimental Procedures) to separate TX-100-soluble and insoluble pools of β-catenin. Samples containing equal amounts of total cellular protein were analyzed for β-catenin expression by immunoblot. The amount of soluble β-catenin is greater in NZO-3/MDCK cells than parental cells at 12 and 24 h, and to a lesser degree at 48 h after the re-addition of calcium. There is no detectable difference in the amount of insoluble β-catenin between parental and NZO-3/MDCK cells.


A
P
NZO-3
6 12 24 48
6 12 24 48 S
NZO-3
ZO-3
ZO-1
ZO-2
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E-cad
B
6 12 24 48
P N P N P N P N
Soluble
β-cat
6 12 24 48
P N P N P N P N
Insoluble
β-cat

over the same time period. The expression levels of all other proteins examined did not change over the course of the calcium switch in either parental or NZO-3/MDCK cells. There was also no detectable difference in the expression levels between parental and NZO-3/MDCK cells at any of the time points, indicating that the effects on junctional assembly caused by NZO-3 expression were not due to altered expression of other junctional proteins. We observed a shift in mobility of occludin in both parental and NZO-3/MDCK cells to a higher molecular weight species over time, corresponding to an increase in phosphorylation of this protein as the tight junction assembles, as previously described (Sakakibara *et al.*, 1997).

In epithelial cells, β-catenin is found in two distinct pools: a TX-100-insoluble pool, which comprises the β-catenin molecules which are bound to E-cadherin and linked to the actin cytoskeleton via α-catenin, and a TX-100-soluble pool that represents the non-adherens junction-associated, cytoplasmic pool of free β-catenin (Fagotto *et al.*, 1996; Funayama *et al.*, 1995). Because these TX-100-soluble and -insoluble pools of β-catenin are functionally distinct, we examined the expression levels of β-catenin in equivalent protein loadings of TX-100-extracted parental and NZO-3-expressing cells. Figure 3.10 (part B) shows that at 12 and 24 h after re-addition of calcium there is more soluble β-catenin in NZO-3/MDCK cells relative to parental cells. This difference is present but less pronounced at 48 h. Equivalent samples of the TX-100-insoluble fractions of parental and NZO-3/MDCK cells show no difference in β-catenin levels.

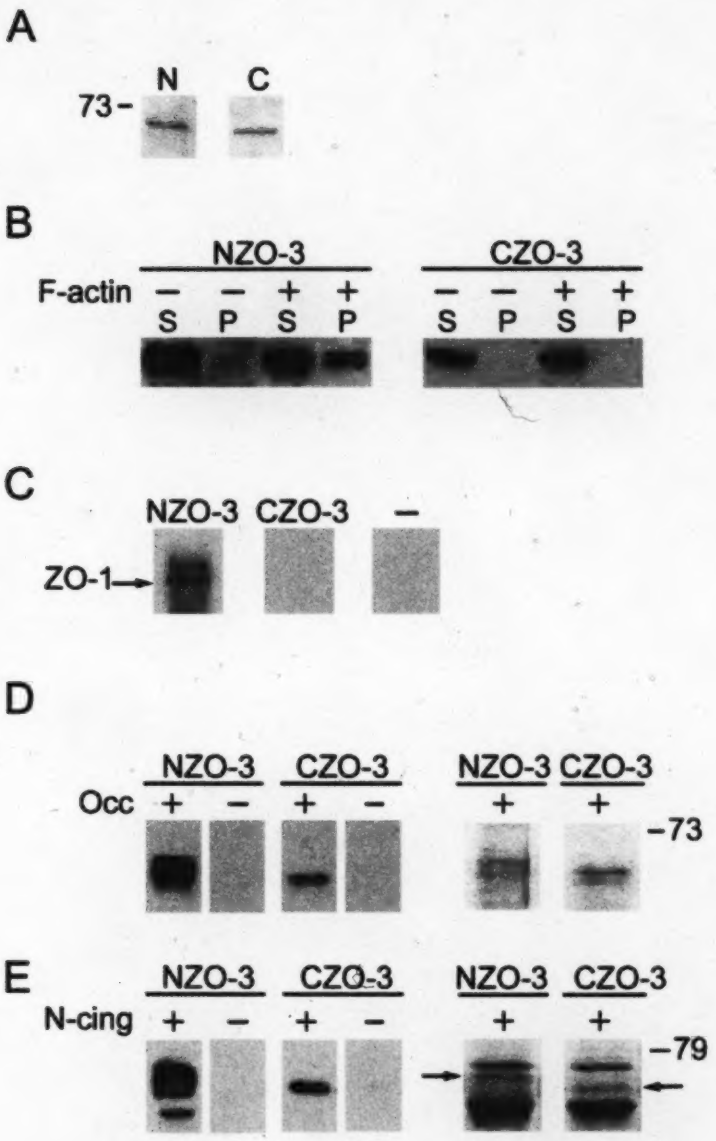
*3.3.7. Protein Binding Analyses*

We determined whether the binding of known ZO-3-interacting proteins could be mapped to either the N- or COOH-terminal halves of the molecule. It has been

previously shown that ZO-3 binds directly to actin filaments *in vitro* (Wittchen *et al.*, 1999; Chapter 2, this thesis). In addition, ZO-3 also binds ZO-1, occludin, and the N-terminus of cingulin, but not to ZO-2 (Haskins *et al.*, 1998; Cordenonsi *et al.*, 1999). Figure 3.11 (part A) shows Coomassie-stained samples of the purified NZO-3 and CZO-3 proteins, demonstrating the equal amounts of these proteins used in subsequent binding experiments. As shown by actin cosedimentation assays in Figure 3.11 (part B), substantial NZO-3 is found in the pellet in the presence of F-actin whereas a much smaller amount is visible in the pellet in the absence of actin, likely due to a low level of fusion protein aggregation. CZO-3 remained in the supernatant fraction in both the presence and absence of actin filaments. Therefore, the actin binding site(s) of ZO-3 reside in the N-terminus of the protein. To map the binding site(s) of ZO-1 on ZO-3, we performed a binding assay using $^{35}$S-labeled *in vitro* transcribed/translated human ZO-1 added to NZO-3 or CZO-3 affinity columns. As shown in Figure 3.11 (part C), NZO-3 retains ZO-1, whereas CZO-3 and the negative control do not. This demonstrates that the N-terminal half of ZO-3 is responsible for binding to ZO-1. This is consistent with previous studies showing that ZO-1 and ZO-2 bind each other via their second PDZ domains (Fanning *et al.*, 1998; Itoh *et al.*, 1999b).

To define the occludin binding site on ZO-3, *in vitro* binding assays were performed using NZO-3 and CZO-3 added to affinity columns containing immobilized GST-occludin or GST alone, and bound proteins were detected by immunoblot (Figure 3.11, part D). Both NZO-3 and CZO-3 were specifically retained on the occludin column. Although the NZO-3 immunoblot reaction is stronger than that for CZO-3, the amounts of NZO-3 and CZO-3 bound to occludin appear approximately equivalent, as

**Figure 3.11.** ***In vitro*** **binding analyses show that NZO-3 binds F-actin and ZO-1 exclusively; both NZO-3 and CZO-3 bind occludin and cingulin.** (A) Coomassie blue–stained gel showing the equivalent amounts of NZO-3 (N) and CZO-3 (C) fusion proteins used in all subsequent binding assays. (B) NZO-3 specifically cosediments with actin filaments; CZO-3 does not. Equivalent amounts of NZO-3 or CZO-3 were centrifuged in the presence (+) or absence (-) of F-actin. Stoichiometrically equivalent aliquots of supernatants (S) and pellets (P) were analyzed by immunoblot. NZO-3, but not CZO-3, is found in the F-actin pellet. (C) NZO-3 binds ZO-1; CZO-3 does not. 35S-labeled *in vitro*–transcribed/translated ZO-1 was incubated with affinity resin containing equal amounts of NZO-3 or CZO-3. The 6-histidine tag plus 36 nonspecific amino acids served as negative control (-). As detected by autoradiogram, ZO-1 is retained by the NZO-3-containing resin and does not bind to CZO-3 or the negative control peptide. (D) Both halves of ZO-3 bind a similar amount of occludin. NZO-3 and CZO-3 were incubated with affinity resin containing immobilized GST-occludin (+), or GST alone (-). Bound protein was eluted with glutathione and analyzed by immunoblot with anti–ZO-3 antibodies (first four lanes) or Coomassie blue staining (last two lanes). Both NZO-3 and CZO-3 are retained by GST-occludin and not by GST alone. The Coomassie blue–stained lanes show that approximately equal amounts of NZO-3 and CZO-3 (arrows) are bound. (E) Both halves of ZO-3 bind to the N-terminal head region of cingulin. NZO-3 or CZO-3 were added to an affinity column containing the N-terminal head region of cingulin (N-cing) fused to GST (+) or GST alone (-). Bound fractions were immunoblotted with antibodies specifically recognizing NZO-3 or CZO-3 (first four lanes) or stained with Coomassie blue (last two lanes). Both NZO-3 and CZO-3 are retained on the GST–N-cingulin column but not on GST alone. Coomassie blue staining of the bound fractions shows approximately equal amounts of NZO-3 or CZO-3 (arrows) are bound. The other protein bands visible in the Coomassie blue–stained samples correspond to GST–N-cingulin. Molecular weights indicated at right (kD).


A
N
C
73-
B
NZO-3
CZO-3
F-actin
– – + +
S P S P
S P S P
C
NZO-3 CZO-3 –
ZO-1
D
NZO-3 CZO-3 NZO-3 CZO-3
Occ + – + – + +
-73
E
NZO-3 CZO-3 NZO-3 CZO-3
N-cing + – + – + +
-79

assayed by Coomassie blue staining of the bound fractions (assuming equal sensitivity of Coomassie blue stain for each half of the protein). This indicates that occludin binding domains are found in both the N- and COOH-terminal halves of ZO-3.

It has recently been reported that the N-terminal head and to a lesser degree, the COOH-terminal tail of cingulin bind to full-length ZO-3 *in vitro* (Cordenonsi *et al.*, 1999). Here we determined which half of ZO-3 binds the N-terminal portion of cingulin. Equivalent amounts of NZO-3 or CZO-3 were added to GST-cingulin or GST affinity columns. As shown in Figure 3.11 (part E), both NZO-3 and CZO-3 are specifically retained by cingulin. Coomassie blue staining of the bound fractions revealed no significant difference between the amounts of NZO-3 or CZO-3 binding to cingulin, indicating that both halves of ZO-3 can bind the head region of cingulin. In summary, the binding studies shown in Figure 3.11 reveal that while both halves of ZO-3 bind to occludin and cingulin equally well, F-actin and ZO-1 bind preferentially to NZO-3.

### 3.4 Discussion

In this study we have shown that exogenous expression of the amino-terminal half of ZO-3 delays assembly of both tight junctions and adherens junctions. Analysis of protein expression showed that whereas the overall levels of the junctional proteins ZO-1, ZO-2, ZO-3, occludin, and E-cadherin remain constant during 48 h of junction assembly, NZO-3 expression increases in response to the calcium switch and then returns to stable, steady-state levels over the same time period. This return of NZO-3 expression to steady-state levels is concomitant with recovery of TER and localization of junctional proteins and actin to the membrane. The amount of β-catenin in the TX-100-soluble pool

is also elevated in NZO-3 expressing cells. Finally, *in vitro* binding experiments revealed that the PDZ domain-containing amino-terminal half of ZO-3 binds to ZO-1 and F-actin, whereas both halves of ZO-3 appear to bind occludin and cingulin. These results suggest that a mechanism involving assembly of the actin cytoskeleton at the junctional membrane mediated through ZO-3 and/or ZO-1 underlies the inhibition of junctional assembly by NZO-3.

### *3.4.1. Targeting ZO-3 to the Tight Junction*

The amino-terminal half of ZO-3 contains three PDZ domains, an arginine-rich domain and a proline-rich region. This portion of the molecule correctly localizes to the tight junction, whereas the carboxy-terminal half, which contains the SH3 domain, GUK domain, and acidic region, remains diffusely distributed in the cytoplasm (Figure 3.3). This is in contrast to the findings for ZO-1, where it was shown that the region encompassing the GUK and acidic region is required for correct targeting (Fanning *et al.*, 1998). Furthermore, when just the three PDZ domains of ZO-1 were expressed in MDCK cells, this construct failed to localize to the tight junction (Reichert *et al.*, 2000) and induced an epithelial to mesenchymal transition. Our results indicate that the PDZ domain-containing region of ZO-3 contains functional characteristics distinct from those of ZO-1.

### *3.4.2. Tight Junction Physiology*

Physiological data pertaining to the formation of the tight junction barrier during junctional assembly was obtained by using TER as an indicator of tight junction integrity. Exogenous expression of NZO-3, CZO-3, or FLZO-3 did not alter steady-state TER levels (data not shown; refer to Figure 3.4, 96 h time point). However, when TER was

measured during the time course of *de novo* tight junction assembly in the calcium switch experimental system, it became apparent that expression of NZO-3 exerted a dominant-negative effect. Whereas CZO-3 or FLZO-3 expression did not affect TER recovery after calcium switch compared with untransfected parental MDCK cells, a calcium switch–induced increase in NZO-3 expression caused a significant lag in TER recovery after the re-addition of calcium (Figure 3.4, part A). It was not until 48 h that NZO-3/MDCK cells approached the TER levels of the other cell lines, an effect that temporally correlated with the return of NZO-3 expression to steady-state levels. The fact that steady-state TER levels were not affected by steady-state NZO-3 expression indicates that this construct exerts its effect only at the higher expression levels induced by the calcium switch and/or specifically during the assembly process, although it can not be ruled out that other undetected phenotypic alterations may be occurring.

We used recovery from cD treatment as a second tight junction assembly paradigm, and these experiments corroborated the calcium switch results. When parental cells or cell lines expressing FLZO-3, NZO-3, or CZO-3 were treated with the actin-disrupting drug, all showed the expected immediate drops in TER. However, when cD was washed out, the NZO-3/MDCK cells displayed a lag in TER recovery (Figure 3.4, part B). These results are consistent with the hypothesis that the NZO-3 construct exerts its effects on junction assembly through interactions with the actin cytoskeleton. An unexpected result of NZO-3 expression during the calcium switch experiment was that adherens junction formation, as assessed by localization of E-cadherin (Figure 3.8) and β-catenin (Figure 3.9) to cell borders, was also delayed. The current model of junctional complex assembly in polarized epithelial cells is based on the discovery that E-cadherin-

mediated cell adhesion provides the initial step that must occur before tight junctions can form (Gumbiner *et al.*, 1988). Our data support the novel concept that exogenous expression of a truncated tight junction protein component can exert effects on the adherens junction-associated proteins E-cadherin and β-catenin. The fact that both tight junction and adherens junction proteins are similarly delayed in their assembly at the membrane lends credence to the notion that the two types of junctions share a coordinated assembly process, and that NZO-3 affects one or more steps in this process.

Immunofluorescence localization experiments showed that the tight junction proteins ZO-1, ZO-2, endogenous ZO-3, and occludin, and the adherens junction proteins E-cadherin and β-catenin were mislocalized during the early stages of junction assembly after calcium switch (Figures 3.5, 3.8, 3.9, and data not shown). After overnight incubation in low calcium, junctional proteins were found in large aggregates, which possibly represent collapsed and internalized junctions. It is known that junctional assembly can occur in the absence of protein synthesis, which indicates that the junctional components can be obtained using the pools of pre-existing proteins (Martinez-Palomo *et al.*, 1980). Perhaps these intracellular aggregates are the source of these proteins during *de novo* junctional assembly. In our experiments, the recovery of the typical junctional localization of these proteins at cell borders temporally correlated with TER recovery after calcium switch. The fact that at later time points the cells were able to overcome the inhibitory effect exerted by NZO-3, as determined by the recovery of TER and correct targeting of ZO-1, ZO-2, ZO-3, occludin, E-cadherin and β-catenin to cell-cell contacts, is likely due to the observed return of NZO-3 protein expression to steady-state levels at later time points (Figure 3.10, part A). The mechanism(s) by which

the calcium switch treatment causes NZO-3 expression to increase and then return to stable, steady-state levels is not known, although the effect was observed in repeated experiments with two independent cell lines. Regardless, our results demonstrate that the NZO-3 construct has a dominant-negative effect on junctional complex assembly.

Immunofluorescence staining of the NZO-3 construct was faint at the later time points, likely due to the lower levels of NZO-3 expression. We did not try to boost expression levels with sodium butyrate (which is sometimes used to boost mRNA translation) because such treatment was toxic to the cells after periods longer than overnight, and overnight pulses of sodium butyrate treatment during calcium switch caused TER levels in parental cells to fluctuate significantly (data not shown). In addition, the anti-VSVG antibody only weakly reacted with our epitope-tagged construct in immunofluorescence and exhibited nonspecific nuclear staining, making it difficult to interpret the finer details of NZO-3 localization. However, we were able to determine that NZO-3 colocalizes with ZO-1 at cell borders at more mature tight junctions during calcium switch (Figure 3.6). The NZO-3 construct did not colocalize with ZO-1 in the large intracellular aggregates that were observed in cells lacking cell border staining or with thick bars of ZO-1 at single cell–cell contacts that may represent two adjacent cells beginning tight junction formation (Figure 3.6). Because of the high nonspecific background staining, it is difficult to see whether another pool of NZO-3 exists in the cytoplasm. Based on the data presented here, and by confocal Z-section analysis which shows precise colocalization of NZO-3 and ZO-1 at the tight junction and no overlap of NZO-3 with E-cadherin more basally situated along the lateral membrane (data not

shown), we believe the NZO-3 construct is not found at the adherens junction during the calcium switch experiment.

The involvement of the actin cytoskeleton in maintaining tight junction integrity and regulating permeability has been well documented; this involvement is underscored by the fact that actin has multiple protein binding partners at the tight junction (Itoh *et al.*, 1997; Fanning *et al.*, 1998; Wittchen *et al.*, 1999; Chapter 2, this thesis), which themselves interact in various ways. It can be envisioned that this molecular architecture provides the means by which an actin filament network can be recruited to and organized in a functionally relevant manner at the tight junction. The actin cytoskeleton is also a major structural and functional element of the adherens junction (Farquhar and Palade, 1963; Rimm *et al.*, 1995; Yonemura *et al.*, 1995), and is present in a bundled actin belt around the apical periphery cell at the level of the adherens junction. Interestingly, in NZO-3/MDCK cells, there is a delay in actin recruitment and formation of this perijunctional apical actin ring (Figure 3.7). Because the N-terminal half of ZO-3 is responsible for binding F-actin, this may represent one mechanism whereby expression of this construct affects tight junction and adherens junction assembly.

*3.4.3. Possible Involvement of β-Catenin*

Not only did expression of the N-terminus of ZO-3 alter the distribution of β-catenin, but there was also an increase in the TX-100–soluble pool of signaling-active β-catenin. Presumably the presence of an increased level of the NZO-3 construct at early time points after calcium switch results in a downstream alteration of the E-cadherin/catenins complex at the adherens junction, releasing β-catenin from a cytoskeletal linkage into the TX-100-soluble pool. A corresponding change in the levels of β-catenin in the

insoluble pool is not observed, although any possible change may be masked by the overall high levels of β-catenin present in these samples. Normally cytoplasmic β-catenin levels are strictly regulated via a ubiquitin-mediated proteolysis pathway requiring β-catenin interaction with the cytoplasmic tumor suppressor APC (Aberle *et al.*, 1997). Soluble β-catenin that escapes this targeted proteolysis is capable of translocating to the nucleus where it acts as a transcriptional transactivator in a complex with TCF/LEF family of transcription factors to direct transcription of a variety of genes that promote a proliferative phenotype (Behrens *et al.*, 1996; Huber *et al.*, 1996; Tetsu and McCormick, 1999). Recently, Stewart *et al.* (2000), showed that expression of a mutant signaling-active (soluble) form of β-catenin in MDCK cells caused a delay in the establishment of tight confluent cell monolayers compared with control cells, and the cells appeared more motile and formed less compact colonies when plated at a low density. These results, taken together with our data showing that NZO-3 expression delays TER formation after a calcium switch and results in an increased level of soluble β-catenin in these cells, suggests that NZO-3 might act through β-catenin to exert its effects on epithelial junctional complex formation. At present we do not know if this action is direct or indirect. It is possible that the increased soluble β-catenin in these cells is a secondary result, and that NZO-3 expression exerts its effect at a point further upstream in the Wnt signaling pathway. For example, one cell might be able to initiate a signal through the use of a soluble Wnt factor.

### *3.4.4. Conclusions*

In summary, through these studies we attempted to further elucidate the function of ZO-3 in epithelial cells, including the role it plays in junctional complex assembly and

its protein binding interactions. Exogenous expression of partial constructs is the experimental method we chose to approach the dissection of ZO-3 protein function and tight junction physiology. This study demonstrates that exogenous expression of the PDZ domain-containing amino-terminal half of ZO-3 perturbs both tight junction and adherens junction assembly. Moreover, expression of NZO-3 alters actin dynamics and increases the amount of soluble signaling-active β-catenin. We hypothesize that NZO-3 exerts its effect on junctional assembly via a mechanism that involves its F-actin and ZO-1 binding ability and/or the increase in soluble β-catenin.

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# CHAPTER 4

## NZO-3 Expression Causes Global Changes to the Actin Cytoskeleton in MDCK Cells: A Possible Link Between a Tight Junction Protein and the Rho GTPases

## 4.1. Introduction

The tight junction is the structural element in epithelial and endothelial cells that creates a selectively permeable barrier to the free diffusion of solutes, small molecules and ions through the paracellular pathway. The tight junction is one element of a tripartite junctional complex in epithelial cells, comprised of the tight junction, adherens junction, and desmosomes.

### *4.1.1. Coordinated Assembly of the Tight Junction and Adherens Junction*

A growing body of evidence indicates that the individual junctions within the junctional complex are jointly regulated in their assembly and functional regulation. This hypothesis proposes that the first step in junctional complex formation involves E-cadherin-mediated cell adhesion (Gumbiner *et al.*, 1988), followed by transient ZO-1 localization to the adherens junction before recruitment to the newly formed tight junction (Rajasekaran *et al.*, 1996). However, recent data suggest that this hierarchy of junctional complex assembly is not absolute. Troxell *et al.* (2000) have shown that tight junction assembly was extensive in MDCK cells expressing a mutant E-cadherin protein lacking the extracellular domain required for cell-cell adhesion, suggesting the possibility that tight junctions can assemble in the absence of an initial E-cadherin-mediated cell adhesion event.

Recently, data from our laboratory has added to the notion of cross-talk between the tight junction and adherens junction (Wittchen *et al.*, 2000; and Chapter 3, this thesis). We studied the effects of expressing an amino-terminal truncation mutant of the tight junction protein ZO-3 (NZO-3; contains the 3 PDZ domains) on tight junction physiology and junctional complex assembly. Expression of this construct in MDCK

cells caused a significant delay in TER recovery during a calcium switch experiment, indicating that assembly of tight junction barrier properties was perturbed. Expression of NZO-3 also disrupted the normal recruitment of tight junction proteins and the adherens junction proteins E-cadherin and β-catenin during the early stages of junctional complex formation. While many investigators have shown evidence that disruption of adherens junction components negatively regulates the tight junction, these results show that the opposite can also be true; mutation of a tight junction protein that disrupts tight junction assembly and physiology can also negatively regulate adherens junction assembly.

*4.1.2. The Actin Cytoskeleton during Junctional Assembly and Barrier Function*

During these experiments we also looked at the effects of NZO-3 expression on the actin cytoskeleton, and observed that recruitment of perijunctional F-actin during junctional assembly was similarly delayed (Wittchen *et al.*, 2000; Chapter 3, this thesis). It is well established that there is reciprocity between actin filament integrity and junctional physiology. For example, pharmacological perturbation of the F-actin cytoskeleton by the drug cytochalasin D causes a drop in TER across the cell monolayer (Stevenson and Begg, 1994). Likewise, disassembly of the junctional complex by incubation in calcium-free media disrupts the junction-associated peripheral actin ring (Wittchen *et al.*, 2000).

*4.1.3. The Rho family of GTPases Regulates Actin Cytoskeleton Dynamics*

The Rho family of GTPases, members of the Ras superfamily of small GTPases, are among the signaling molecules involved in dynamic regulation of the actin cytoskeleton. Remodeling the actin cytoskeleton is critical to cell morphology changes, cytokinesis, substrate adhesion, cell spreading, and migration (Kaibuchi *et al.*, 1999). A

key feature of GTPases is their cyclical activation and deactivation by the binding of GTP followed by intrinsic GTPase conversion to GDP, thus allowing them to act as molecular switches. The cycle of activation-deactivation is a process that is regulated by specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs promote the activation of the RhoA GTPases by facilitating the release of GDP from the molecule, allowing subsequent binding of GTP due to the higher cytosolic concentration of GTP compared to GDP (Cerione and Zheng, 1996). GAPs act as negative regulators of the Rho GTPases by stimulating the intrinsic GTPase activity, causing the conversion of GTP to GDP (Hall, 1990).

The Rho GTPase family members RhoA, Rac1, and Cdc42 each play a specific role during cytoskeletal reorganization. RhoA controls the formation of stress fibers and focal adhesions based in part on the observation that microinjection of constitutively active RhoA into fibroblasts induces the formation of stress fibers and focal adhesions (Ridley and Hall, 1992). Furthermore, microinjection of the catalytic domain of Rho-kinase, which is a downstream effector of RhoA, into fibroblasts, also caused the formation of stress fibers and focal adhesions (Amano *et al.*, 1997). In general, the presence of abundant stress fibers and focal adhesions is associated with a non-motile phenotype characterized by strong cell-substratum attachment (Herman *et al.*, 1981; Ridley *et al.*, 1995; Nobes and Hall., 1999).

Both Rac and Cdc42 activity is linked to the formation of membrane protrusions at the leading edge of migrating cells. Rac promotes the formation of lamellipodia, which are broad actin filament-based extensions of the plasma membrane (Ridley at el., 1992). Cdc42 promotes filopodia formation (Nobes and Hall, 1995), which is another

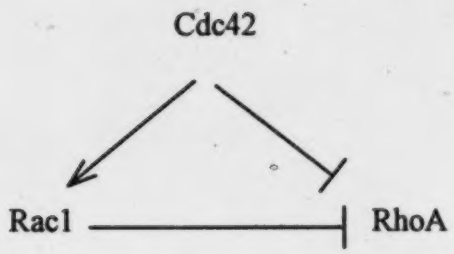
actin-based protrusion found at the cell periphery. Both structures are characteristics of membrane ruffling, which is the dynamic movement of membrane protrusions at the free-edge of cells involved in cell spreading and migration. Cell spreading and increased motility is associated with the presence of lamellipodia and filopodia at the cell periphery concomitant with a decrease in the amount of stress fibers and focal adhesions (Ridley *et al.*, 1995).

Not only do the various Rho GTPases have specific effects on the types of actin structures formed in the cell as described above, but there is also evidence that they can affect the activity of each another. Sander and colleagues (Sander *et al.*, 1999) theorize that there is a hierarchical organization of Rho GTPase activity, with Rac activity down-regulating RhoA activity. Cdc42 activity can also downregulate RhoA activity either by direct inhibition of RhoA, or indirectly by activation of Rac (Sander *et al.*, 1999) (Figure 4.1, part A). Furthermore, investigators have discovered a link between oncogenic activation of Ras and decreased Rac activity via Raf/MAPK signaling and transcriptional down regulation of Tiam1, a Rac activator (Michiels *et al.*, 1995), which releases the inhibition of RhoA activity and leads to an epithelial-mesenchymal transition (EMT) (Zondag *et al.*, 2000) (Figure 4.1, part B).

*4.1.4. The Rho GTPases and Junctional Complex Assembly and Function*

There is also a growing body of evidence that supports a role for the Rho GTPases in junctional complex assembly and physiological regulation in epithelial cells. One of the first papers to connect tight junction physiology with RhoA activity showed that treatment of epithelial cells with C3 transferase, an inhibitor of RhoA, decreased TER and increased paracellular flux, indicating that tight junction barrier properties were

A




B

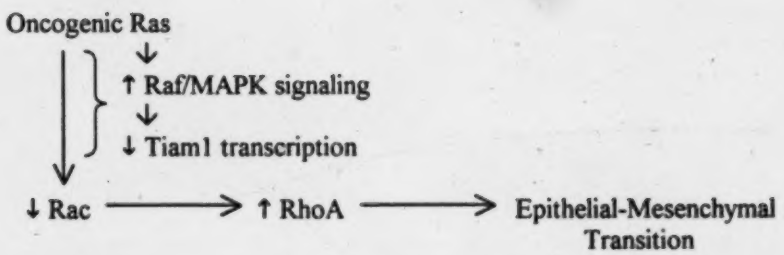



**Figure 4.1. Schematic representation of some signaling pathways involving the RhoA GTPases.** (A) Hierarchical organization of the three GTPases RhoA, Rac1, and Cdc42. Rac and Cdc42 activity inhibits RhoA directly, and Cdc42 activity can activate Rac1. (B) Constitutive Ras activation causes decreased Rac1 activity through Raf/MAPK signaling which causes decreased Tiam1 transcription. Tiam1 is a Rac activator (GEF), thus decreased Tiam1 levels lead to inhibited Rac activity. Since Rac activity is lower, RhoA activation is increased which can lead to an epithelial-mesenchymal transition.

compromised (Nusrat *et al.*, 1995). Inhibition of RhoA also caused a disruption of perijunctional actin formation, and caused a redistribution of ZO-1 and occludin away from the membrane (Nusrat *et al.*, 1995). More recently, Jou *et al.* demonstrated that expression of either dominant negative or constitutively active RhoA and Rac in MDCK cells reduced TER and perturbed tight junction fence function, as indicated by the unrestricted diffusion of membrane lipids from the apical to the lateral membrane (Jou *et al.*, 1998).

The assembly of adherens junctions also seems to involve the RhoA pathway. Inhibition of p160ROCK (a downstream effector of RhoA) prevents localization of E-cadherin in addition to the tight junction proteins ZO-1 and occludin to the membrane during junctional complex assembly (Walsh *et al.*, 2001). Both RhoA and Rac1 activities are required for E-cadherin to be localized to the adherens junction in MDCK cells (Braga *et al.*, 1997). Furthermore, during adherens junction assembly Rac1 is co-localized with E-cadherin at sites of cell-cell contact, and translocates to the cytoplasm if junctions are disrupted by removing calcium (Nakagawa *et al.*, 2001). This group and others also demonstrate that E-cadherin mediated cell-cell adhesion stimulates Rac1 activation (Nakagawa *et al.*, 2001; Noren *et al.*, 2001).

Because of the close association of actin (the perijunctional ring) with the junctional complex of epithelial cells, it is becoming apparent how Rho family members can affect junction physiology via cytoskeleton regulation. As mentioned in Chapter 1, tight junction permeability might be regulated by contraction of the perijunctional actin ring. This contraction force could subtly increase the tension generated between opposing cell surfaces and partially open the tight junction. One interesting hypothesis is

that rapid cycling of RhoA and Rac between active and inactive forms fine-tunes the tension subjected on the peripheral actin ring, thus regulating the degree of tight junction permeability (Jou *et al.*, 1998).

*4.1.5. P120 catenin: Dual Roles in Adhesion and Motility*

Another protein known to play a role in cytoskeletal changes linked to both cell-cell adhesive junctions and cell motility is p120 catenin. P120 catenin is a member of the armadillo family of proteins which also includes β-catenin. Similarly to β-catenin, p120 catenin is found in two distinct subpopulations within the cell, an insoluble pool bound to E-cadherin at the adherens junction and a soluble cytoplasmic pool. Like β-catenin, when p120 catenin is not bound to E-cadherin it enters the soluble pool where it can then be translocated into the nucleus and bind to a transcription factor, Kaiso (Daniel and Reynolds, 1999).

P120 catenin has dual functions within the cell that directly correlate to its localization. When bound to E-cadherin at the adherens junction, p120 catenin promotes cell adhesion, perhaps by cadherin clustering (Yap *et al.*, 1998). However, increased cytoplasmic p120 catenin causes increased cell motility via modulation of the activity of the Rho GTPases. Noren et al. (Noren *et al.*, 2000) have shown that increasing the soluble pool of p120 catenin results in disassembly of focal adhesions and stress fibers, and correspondingly decreases RhoA activity while increasing Rac and Cdc42 activity. They further provide a direct link between p120 catenin and the Rho GTPase family proteins by demonstrating that p120 catenin directly binds to Vav-2, an activator (GEF) of Rac and Cdc42. Other investigators have confirmed this result and show *in vitro* that p120 catenin inhibits GDP dissociation from RhoA (Anastasiadis *et al.*, 2000). These

studies suggest a model where p120 catenin shuttles between a cadherin-bound state that modulates cell adhesion, and a cytoplasmic pool where it affects the activity of RhoA GTPase family members and increases cell motility by interacting with an exchange factor (Noren *et al.*, 2000).

*4.1.6. Chapter Summary*

Our findings that actin remodeling during junctional assembly was affected by expression of a mutant tight junction protein (Wittchen *et al.*, 2000; and Chapter 3) are novel and warranted further investigation. In this chapter, I have examined other changes in actin cytoskeleton dynamics in NZO-3/MDCK cells. The results presented here identify a global alteration of actin dynamics in NZO-3-expressing cells, not only during junctional assembly as detailed in Chapter 3. I show here that expression of NZO-3 in MDCK cells decreases the amount of stress fibers and focal adhesions in the cell, and increases the rate of cell migration. In addition, I have begun to explore the molecular mechanism underlying these changes and provide evidence for the involvement of the GTPase RhoA and possibly p120 catenin. The information obtained from these studies sheds light on the mechanisms of cytoskeleton reorganization by the Rho family of GTPases during junctional assembly and wound healing, and how tight junction elements might influence these processes.

## 4.2. Materials and Methods

*4.2.1. Cell lines*

Parental MDCK (untransfected), NZO-3/MDCK, CZO-3/MDCK, and FLZO-3/MDCK cell lines have been described previously (Haskins *et al.*, 1998; Wittchen *et al.*,

1999; 2000). Immunofluorescence experiments, wound healing assays, and RhoA activity assays were repeated with two independent NZO-3/MDCK cell lines (clones D5 B6), and two independent CZO-3/MDCK cell lines (data from one line will be shown).

*4.2.2. Immunohistochemistry*

*Subconfluent monolayers; F-actin staining: MDCK* cell lines were plated on collagen-coated coverslips, and allowed to grow until they reached a subconfluent density of ~50%. Cells were fixed and permeabilized using 2.5% paraformaldehyde on ice for 30 min followed by incubation with 0.2% TX-100 /TBS+ for 5 min at room temperature (RT). The coverslips were blocked for 15 min RT in TBS+/BLOTTO (5% skim milk powder in TBS + $Ca^{2+}$ and $Mg^{2+}$), followed by 1 h incubation with rhodamine-phalloidin (Sigma-Aldrich). After washing 3 x 5min with TBS + $Ca^{2+}$ and $Mg^{2+}$, coverslips were mounted on glass slides using an anti-bleaching mounting media, (VectaShield; Vector Laboratories), and viewed with a Ziess Axioskop fluorescence microscope (Carl Zeiss, Inc.).

*Wounded monolayers: Actin/Vinculin:* Wounded monolayers (see below) were fixed 3 hrs post-wounding as above, and co-stained with anti-vinculin antibody (Zymed), and FITC-phalloidin (Sigma-Aldrich) to detect actin filaments. Vinculin localization was visualized with a rhodamine anti-mouse secondary antibody.

*4.2.3. Wound Healing Assays*

To measure migration ability, *in vitro* wound healing assays were performed. Briefly, cells were plated at varying densities, and dishes that had just reached confluence were used. Two linear wounds were scratched in each dish of cells using a p200 pipet tip. Using a phase microscope attached to a MD2 microscope digitizer (AccuStage, MN,

USA), which measures stage position, the width of the wound at two different points was measured over a 6 h time period. The average migration rate was calculated by taking the total distance migrated (μm) divided by the total time (hr), and expressed as μm/hr. For each cell line, two different wounds were made per dish, and width was measured at two points per wound (N=4). Data shown are representative of 6 independent experiments.

*4.2.4. RhoA Activity Assays*

We performed Rho activity assays as previously described (Ren *et al.*, 1999; Noren *et al.*, 2000) with slight modifications. The GST-RBD construct (amino acids 7-89 from rhotekin) was kindly provided by Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC). Briefly, an ~80% confluent 10 cm dish of cells was washed in ice-cold TBS and lysed by scraping in 1 ml RIPA buffer [50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, and protease inhibitors]. Lysates were clarified by centrifugation at 12 000 *g* at 4°C for 5 min, and equal volumes of lysates were incubated with 30 μg GST-RBD beads at 4°C with rotation for 30 min. An aliquot of lysate was reserved for analysis of total RhoA. Beads were washed four times with 1 ml Buffer B [TBS + 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, and protease inhibitors]. The bound fraction (active RhoA) was analyzed by resuspending the beads in 2X GSB, boiling 5 min, and running on SDS-PAGE. Active RhoA (bound fraction) and total RhoA were analyzed by blotting with anti-RhoA antibodies. The results were quantified by scanning densitometry of western blots from 4 independent experiments. RhoA activity was determined by taking the ratio of the amount of RhoA sedimented by the GST-RBD beads relative to the total amount of

RhoA in the whole cell lysate in order to compare the activity of RhoA from different samples.

*4.2.5. GST Pulldown Assays*

GST fusion proteins were expressed and purified as previously described (Wittchen *et al.*, 1999; Wittchen *et al.*, 2000). Equivalent amounts (determined by Coomassie blue staining) of GST-FLZO-3, GST-NZO-3, GST-CZO3, or GST alone were bound to glutathione-sepharose beads. Cell lysate was made by lysing cells in RIPA Buffer, and an equal amount of lysate was added to each batch of affinity resin. Batch binding was carried out overnight at 4°C with rotation. Beads were then washed 4x with Buffer B plus protease inhibitors, resuspended in 2x GSB, boiled 5 min, and loaded for SDS-PAGE.

**4.3. Results**

Our previous observation that NZO-3 expression induced a delay in actin recruitment to the junctional membrane during junctional assembly prompted us to look at other aspects of actin dynamics in these cells. One of the first observations we made was that NZO-3/MDCK cells contained fewer actin stress fibers than parental untransfected cells or CZO-3-expressing cells. Figure 4.2 shows NZO-3/MDCK cells and parental MDCK cells plated at subconfluent density, and co-stained with phalloidin to visualize F-actin and vinculin (as a marker for focal adhesions). Thick actin stress fiber bundles are abundant in parental cells, while NZO-3/MDCK cells have almost a complete absence of basally-located stress fibers. In addition, the limited stress fibers present are thinner and less bundled. The perijunctional apical actin ring of NZO-3/MDCK cells is

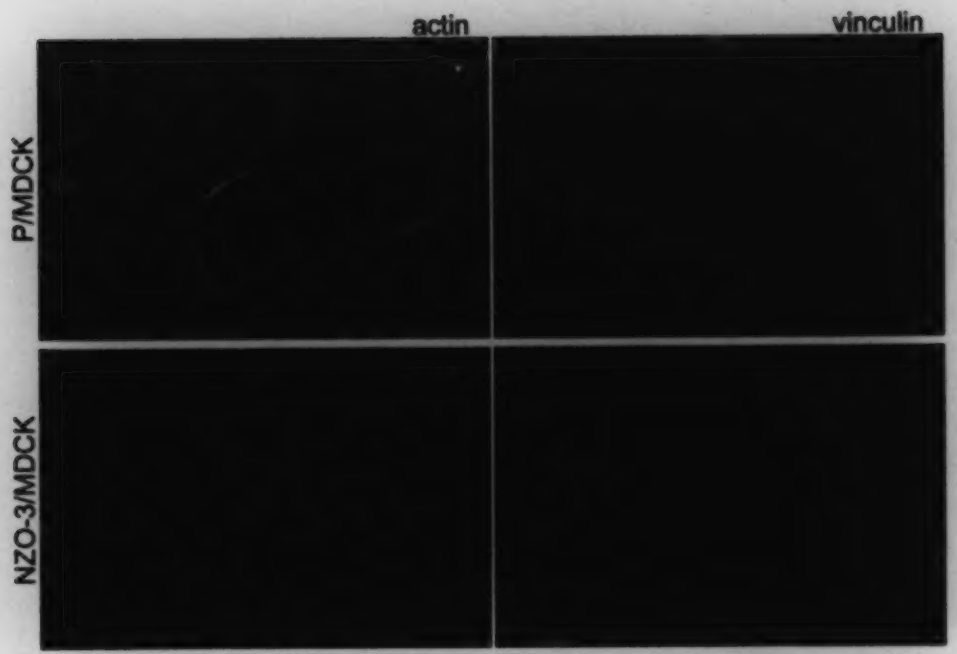


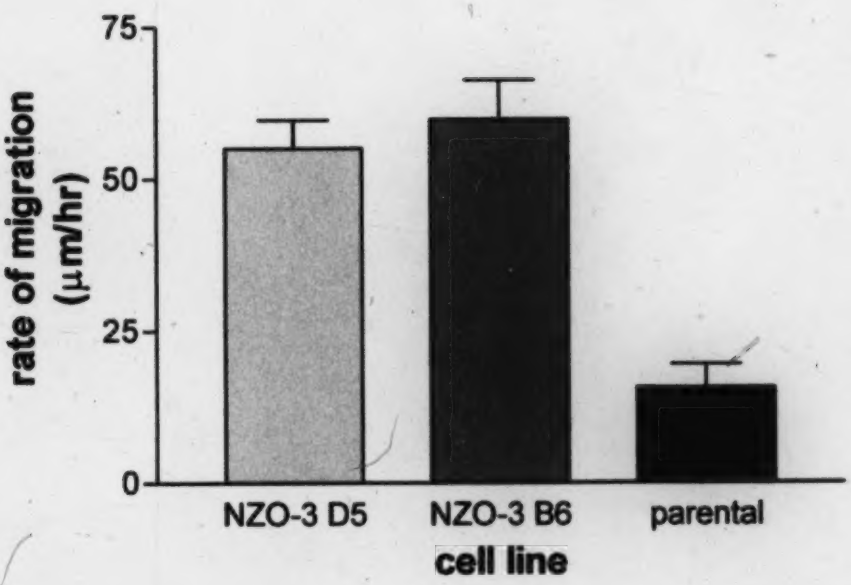

**Figure 4.2. NZO-3/MDCK cells have fewer stress fibers and focal adhesions than untransfected parental MDCK cells.** NZO-3/MDCK cells and parental MDCK cells (P/MDCK) were grown to subconfluency, then fixed and permeabilized with 2.5% paraformaldehyde/0.5% TX-100. Cells were costained with FITC-phalloidin to visualize F-actin, and anti-vinculin antibodies (rhodamine) as a marker for focal adhesions. NZO-3-expressing cells show a striking decrease in the amount of stress fibers, and a decrease in number and size of focal adhesion complexes.

normal compared to parental untransfected cells. The vinculin co-staining revealed that NZO-3/MDCK cells also had fewer and smaller focal adhesions (Figure 4.2). Cells expressing CZO-3 presented abundant stress fibers, similar to parental untransfected cells (data not shown).

Based on this reduced stress fiber and focal adhesion phenotype, we hypothesized that NZO-3/MDCK cells might migrate faster than parental cells due to weaker attachment to the substratum. Many studies have demonstrated an inverse correlation between the presence of actin stress fibers and increased motility (Herman *et al.*, 1981; Ridley *et al.*, 1995; Nobes and Hall., 1999). We therefore performed *in vitro* wound healing assays to test this theory. Just confluent cell monolayers were manually scratched with a pipet tip to create a linear wound, and the width of the wound at a specific location was measured over a 6 hour period. The total migration rate (μm/hr) for two independent NZO-3/MDCK cell lines (B6 and D5) was compared to parental untransfected cells, and we found that both NZO-3-expressing cell lines migrated ~4-fold faster than parental MDCK cells (Figure 4.3, part A). As a control, migration rate of CZO-3/MDCK cells were compared to parental MDCK cells. As shown in Figure 4.3 (part B), there was no significant difference in migration rate between these two cell lines which correlated well with their having similar stress fiber phenotypes (Figure 4.2 and data not shown). When wounded monolayers were stained with phalloidin to visualize actin, not only are the number of stress fibers reduced, but it is also apparent from studying many stained coverslips that there is less F-actin at the wound edge of NZO-3/MDCK cells compared to parental cells (One representative field shown in Figure 4.4).

**Figure 4.3. NZO-3/MDCK cells migrate faster than untransfected parental MDCK cells and CZO-3/MDCK cells in an *in vitro* wound-healing assay.** A. Cell monolayers that have just reached confluency were manually scratched with a pipet tip to create a linear wound. Using a microscope/digitizer, wound width was measured at the same XY coordinates on two separate wounds (n=4) per dish. The average distance migrated over a six hour period is plotted in μm/hr for two separate stable NZO-3-expressing cell lines (D5 and B6) and parental MDCK cells. Graph representative of 6 independent experiments. B. In a separate experiment, the migration rate of CZO-3/MDCK cells is not significantly different than untransfected parental cells. There is some experimental variation of the migration rate of parental MDCK cells (compare part A to part B) because the experiments were not performed at the same time. Graph representative of 3 independent experiments (n=6).

A


75
50
25
0
rate of migration (μm/hr)
NZO-3 D5
NZO-3 B6
parental
cell line

B

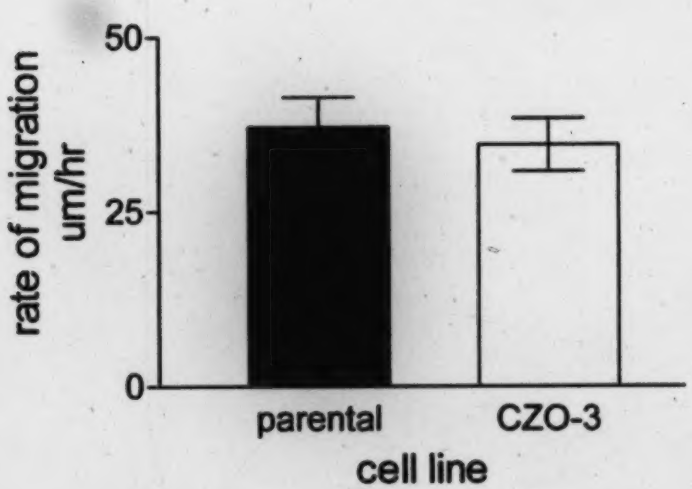

50
25
0
rate of migration um/hr
parental
CZO-3
cell line

**Figure 4.4. Wounded NZO-3/MDCK cell monolayers have less F-actin staining at the free edge of the wound compared to untransfected parental cells.** Wounded monolayers were stained with rhodamine-phalloidin to visualize F-actin. Parental MDCK cells show intense F-actin staining at free-edge of cells adjacent to the wound (arrowheads). The amount of F-actin at the wound edge is reduced in MDCK/NZO-3 cells (arrowheads).

These results identify a more global effect on the actin cytoskeleton caused by NZO-3 expression in MDCK cells, not only during junctional assembly as shown previously (Wittchen *et al.*, 2000). Pursuing the possible mechanism whereby actin cytoskeleton dynamics during junctional assembly and cell migration are affected, we hypothesized that the Rho family of GTPases might be involved. As discussed previously, members of this family are known to be crucial regulators of dynamic actin cytoskeletal reorganization events, and strong evidence exists that RhoA activity is responsible for the formation of stress fibers and focal adhesions (Ridley and Hall., 1992). We therefore reasoned that a possible mechanism underlying the phenotype of NZO-3-expressing MDCK cells would be decreased RhoA activity.

The first parameter we looked at was total RhoA protein content from NZO-3/MDCK, parental MDCK, or CZO-3/MDCK cells. We found no difference in the total amount of RhoA from whole cell lysates of NZO-3/MDCK cells compared to parental and CZO-3/MDCK cells (Figure 4.5). To determine the level of Rho activity, we used an affinity precipitation technique which specifically pulls out active (GTP-bound) RhoA from the pool of total Rho. Previously, Rho GTPase activity could only be inferred based on the presence of characteristic cytoskeleton features like stress fibers as a phenotypic readout. More recently, Ren *et al.*, (1999) developed an assay that can determine the activity of RhoA in cells biochemically. This assay takes advantage of the specific binding of active, GTP-bound RhoA, but not the GDP-bound form to a fusion protein construct comprised of the Rho binding domain of rhotekin, a downstream effector of RhoA, fused to GST (GST-RBD). A representative western blot of the active RhoA fraction versus the total RhoA from the same lysate is shown in Figure 4.6A.

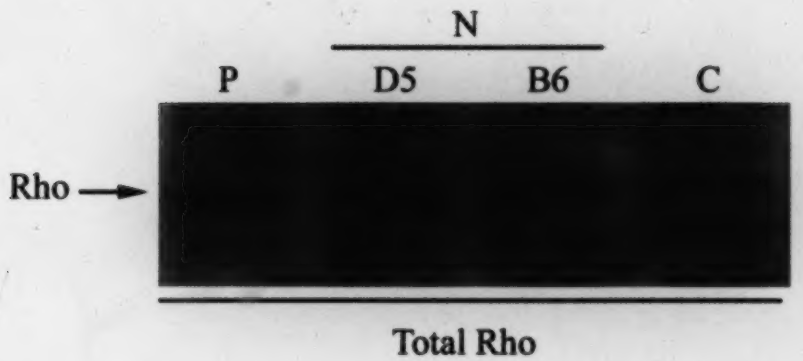



**Figure 4.5. The total amount of RhoA is the same in NZO-3/MDCK cells versus parental MDCK cells and CZO-3/MDCK cells.** Equivalent amounts of cell lysate was loaded in each lane, and the western blot was probed with anti-RhoA antibodies. There is no significant difference in the amount of total RhoA in each cell line.

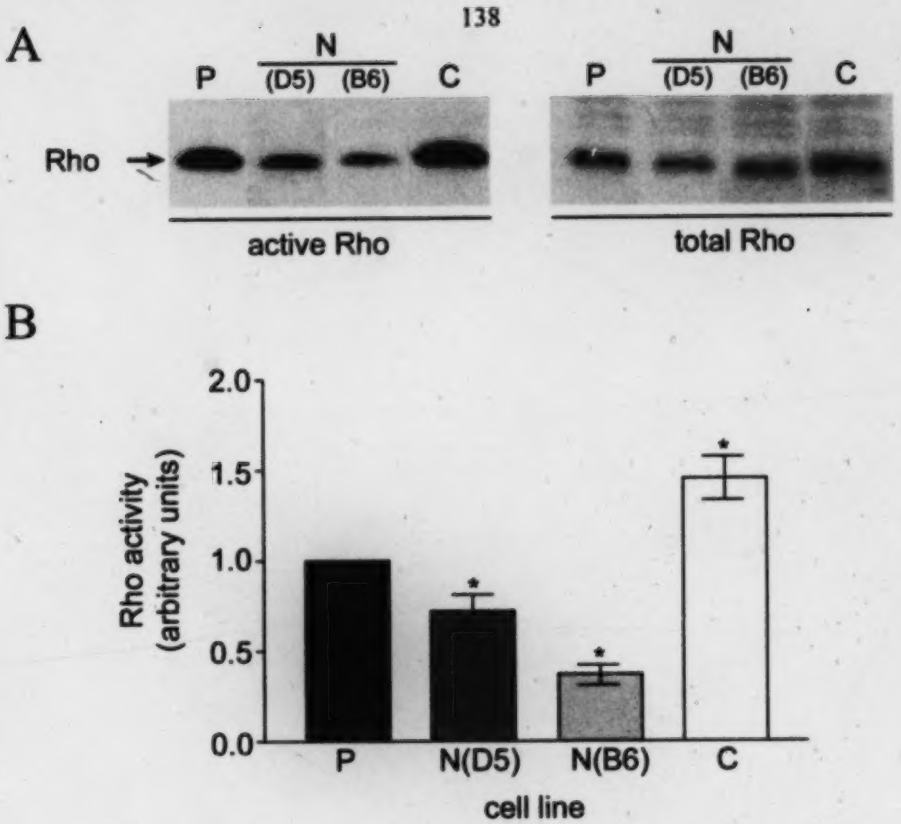



**Figure 4.6. NZO-3/MDCK cells have lower RhoA activity than untransfected parental cells and CZO-3/MDCK cells.** Cells were serum-deprived for 16 hours prior to RhoA activity assays. A. A representative immunoblot shows the amount of active RhoA that was retained on GST-RBD affinity beads (left), and total lysate (right; these samples are not loaded for equivalent protein). B. Rho activity was quantitatively compared using data from 4 independent experiments and plotted ± SEM. A ratio of active Rho/total Rho was used to normalize according to the amount of total Rho. Rho activity in parental MDCK cells was arbitrarily set to a value of 1.0. Two independent MDCK/NZO-3 cell lines (D5, and B6) had significantly lower Rho activity ($p \leq 0.05$) compared to parental MDCK cells. CZO-3/MDCK cells had significantly higher Rho activity compared to parental MDCK cells, although the rate of migration was similar (Figure 4.3).

Two independent NZO-3/MDCK cell lines (D5 and B6) show less active RhoA bound to the GST-RBD beads compared with parental cells. The results were quantified by densitometry of western blots, and the relative activity of RhoA was plotted as a ratio of active RhoA relative to the total amount of RhoA in each sample (Figure 4.6B). Both NZO-3/MDCK cell lines had a significant decrease in the amount of active RhoA compared to parental cells ($p<0.05$). CZO-3/MDCK cells had significantly higher RhoA activity compared to parental cells, even though there was no difference in the number and thickness of stress fibers, and the rate of migration during wound closure was not significantly different (Figure 4.3, part B).

We next performed binding experiments from whole cell lysates to attempt to identify a physical link between the RhoA signaling pathway and the tight junction protein ZO-3. The first question we asked was: does NZO-3 interact with any members of the Rho family of GTPases? Using NZO-3 expressed as a GST fusion protein, we performed GST pull-down experiments with MDCK whole cell lysates, and probed the bound fraction with antibodies to RhoA, Rac, or Cdc42. Figure 4.7 shows that NZO-3 does not interact with Rho, Rac, or Cdc42. *In vitro* binding studies to determine if the COOH-terminal half of ZO-3 interacts with any of these proteins are in progress. However, we were able to identify two novel protein interactions using this GST pull-down technique. Approximately equivalent amounts of GST only, FLZO-3, NZO-3 or CZO-3-GST fusion proteins (as assessed by Coomassie blue staining) were bound to glutathione-sepharose beads and an equal amount of MDCK lysate was allowed to bind overnight. Immunoblotting the bound fraction with an anti-AF-6 antibody revealed that the full-length ZO-3 construct binds to the Ras-effector protein AF-6 (Yamamoto *et al.*,

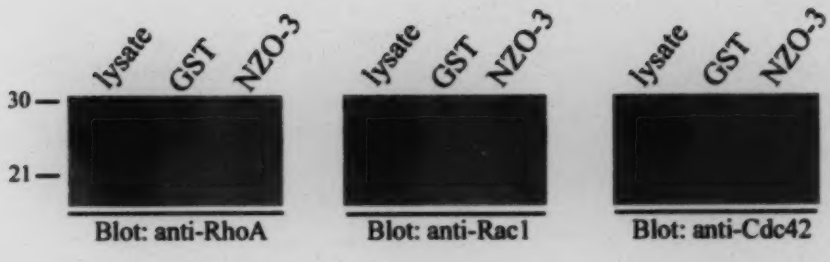



**Figure 4.7. NZO-3 does not interact with RhoA, Rac, or Cdc42 from MDCK lysates.** MDCK lysate was added to affinity columns containing immobilized GST-NZO-3 or GST alone, and the bound fractions were immunoblotted with anti-RhoA, anti-Rac, and anti-Cdc42 antibodies. Presence of each GTPase in the lysate was confirmed by immunoblotting a sample aliquot of lysate. None of the GTPases tested were retained by NZO-3 or GST alone.

1997) and that the COOH-terminal half of ZO-3 appears to contain the binding region (Figure 4.8). NZO-3 shows negligible binding to AF-6. We also determined if any of our ZO-3 constructs could bind to the protein p120 catenin, a member of the catenin family of proteins that is known to play a role in cytoskeletal changes linked to cell adhesion and cell motility (Braga, 2000). We found that p120 catenin binds to full-length ZO-3 and CZO-3, with very little binding to NZO-3 (Figure 4.8).

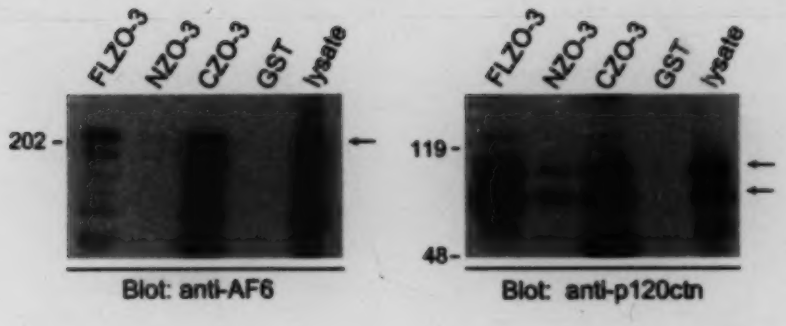



**Figure 4.8. ZO-3 binds to the proteins AF-6 and p120 catenin from whole cell lysates, and the binding region is in COOH-terminal half of ZO-3.** Equivalent amounts of full-length ZO-3 (FLZO-3), NZO-3, CZO-3 or GST alone were immobilized on glutathione-sepharose beads; MDCK lysate was added and allowed to bind overnight. The bound fraction was immunoblotted with anti-p120 catenin or anti-AF-6 antibodies. Both p120 catenin and AF-6 bind to FLZO-3 and CZO-3, with negligible binding to NZO-3, and not to GST alone.

**4.4. Discussion**

In this study, we have exogenously expressed partial constructs of the tight junction protein ZO-3 in MDCK epithelial cells to help elucidate the function of this protein and obtain information about the molecular organization of the junctional complex. In addition to our previous findings that expression of an N-terminal ZO-3 construct (NZO-3) in MDCK cells delays both tight and adherens junction assembly and delays recruitment of F-actin to the perijunctional actin ring (Wittchen *et al.*, 2000), we now show that there are more global effects on F-actin dynamics within these cells. We show here that NZO-3-expressing cells have fewer actin stress fibers and fewer and smaller focal adhesions than untransfected parental cells or cells transfected with the COOH-terminal half of ZO-3. NZO-3-expressing cells also migrate faster than parental MDCK cells in a wound-healing assay. As the Rho GTPases are known to be important regulators of actin cytoskeletal dynamics, we explored the potential relationships between NZO-3 expression and Rho GTPase activity. NZO-3-expressing cells have lower RhoA activity compared to parental MDCK cells and CZO-3/MDCK cells. We also demonstrate that CZO-3 interacts with the Ras target protein AF-6 as well as p120 catenin from MDCK whole cell lysates.

*4.4.1. NZO-3 Expression Affects the Actin Cytoskeleton and Increases Cell Migration*

Our observation that NZO-3/MDCK cells have fewer stress fibers compared with parental untransfected MDCK cells (Figure 4.2) was the observation that initiated this investigation. The fact that this was observed in multiple clonal cell lines expressing NZO-3 and that the stress fibers of CZO-3/MDCK cells are indistinguishable from parental cells indicates that the observation in NZO-3/MDCK cells is specific. Since the

actin cytoskeleton is tethered to focal adhesions via actin stress fiber bundles, it is not surprising that the number and size of focal adhesions is also diminished in NZO-3/MDCK cells (Figure 4.2). The meaning of the observation that NZO-3/MDCK cells have less actin staining at the free edge of the wound compared with parental MDCK cells (Figure 4.4) is unclear, since migrating cells generally have a prominent F-actin condensation in the leading edge lamellipodia (Waterman-Storer *et al.*, 1999). It could be that although there is less F-actin at the leading edge in NZO-3/MDCK cells, the F-actin that is present might be more dynamic with a more rapid turnover, thus promoting membrane protrusive activity.

The phenotype of fewer actin stress fibers and focal adhesions suggested to us that NZO-3/MDCK cells might be more motile. There is a well-established correlation between cell motility and reduced actin stress fibers and focal adhesions, and these F-actin structures are typically considered characteristics of a non-motile state (Herman *et al.*, 1981; Ridley *et al.*, 1995; Nobes and Hall., 1999). The wound-healing assays we performed are one way of measuring the migratory behavior of cells. These assays can be considered as *in vitro* models for physiological processes that occur during development (e.g. gastrulation, neurulation). During wound closure cells migrate to fill the empty region, sometimes in connection with each other, and sometime individual cells break off to migrate on their own. The former condition typifies the cell migration of epithelial cells observed in this study, where there is coordinated migration of a cell sheet and cell-cell interactions are maintained.

*4.4.2. Involvement of the Rho Family of GTPases*

Since the Rho family of GTPases are classical mediators of actin cytoskeleton remodeling during events such as cell migration, it was logical to determine if there were differences in their activity in the NZO-3-expressing cells. We looked at RhoA activity because of its well-established role in stress fiber and focal adhesion formation. It is important to note that total RhoA protein levels were the same in all cell lines (Figure 4.5), so any difference we observed has to be attributed to the activation state of the protein. We found that RhoA activity was lower in NZO-3/MDCK cells compared to parental MDCK cells and CZO-3/MDCK cells (Figure 4.6). Two independent NZO-3/MDCK cell lines were used to confirm this observation, and both cell lines showed significantly decreased RhoA activity when compared to parental MDCK cells. Interestingly, this difference was only seen under serum-free conditions, and not in the presence of 5% serum: We hypothesize this is due to higher basal activation of RhoA in the presence of serum that masks small but physiologically relevant differences. That we saw the lack of stress fibers and focal adhesions in the presence of serum, but we do not see decreased RhoA activity under these conditions is not an atypical observation (Noren *et al.*, 2001). Due to technical reasons we were unable to ascertain whether NZO-3/MDCK cells have fewer stress fibers under serum-free conditions, because the cells lift off from the coverslips during processing for immunofluorescence. I would predict that under serum-starved conditions all of our cell lines would show a reduction in the amount of actin stress fibers, and that it would be more pronounced in NZO-3/MDCK cells.

It is also interesting to note that CZO-3/MDCK cells had significantly higher RhoA activity compared to parental cells, even though there was no difference in the

amount of stress fibers (data not shown) between the two cell lines, and the rate of migration during wound closure was similar (Figure 4.3, part B). It is likely that there is a maximum threshold level of RhoA activation, and any activity higher than this will not have any additive effects on the cytoskeleton. Perhaps the CZO-3/MDCK cells have exceeded this threshold of RhoA activity, and the cellular machinery responsible for stress fiber formation is saturated. Alternatively, negative-feedback mechanisms may exist to counteract the phenotype produced by excessive RhoA signaling. In this regard, it will be interesting to do Rac and Cdc42 activity assays in these cells, because increased activity of these GTPases may have an inhibitory effect on RhoA (Sander *et al.*, 1999).

*4.4.3. Linking ZO-3 with the RhoA signaling Pathway: Binding Interactions*

Identifying novel binding partners for ZO-3 in general will provide information about its cellular function, and especially in context of the observations presented here it might provide information on the molecular mechanism underlying the phenotypes we observe in NZO-3/MDCK cells. One potential mechanism by which NZO-3 expression could decrease the activity of RhoA is by interacting with the GTPase and either inhibiting its activity directly or indirectly by preventing it from binding to its GEFs. We tested whether NZO-3 could bind to RhoA from MDCK whole cell lysate and could not detect an interaction (Figure 4.7). It is also possible that the NZO-3 interaction with the RhoA GTPases is transient or with such low affinity that binding is undetectable in the *in vitro* binding assays performed. Alternatively NZO-3 might interact with a Rho GDP-dissociation inhibitor, keeping RhoA in an inactive (GDP-bound) state, or a Rho GTPase family exchange factor.

By performing *in vitro* binding assays, we found that FLZO-3 and CZO-3, but not NZO-3, interact with the Ras target AF-6. It has been shown previously that the Ras-binding domain of AF-6 interacts directly with the tight junction protein ZO-1, and activated Ras competes with ZO-1 for binding to AF-6 (Yamamoto, 1997). When epithelial cells are transformed with Ras, they acquire a fibroblastic, depolarized phenotype (Schoenenberger *et al.*, 1991). In conjunction with this, a correlation between Ras activation and junctional complex disruption exists (Mercer, 2000). In Ras-transformed MDCK cells, TER is low, indicating disrupted barrier properties, and occludin, claudin-1 and ZO-1 were not localized to the cell membrane at tight junctions. TER and localization of these proteins to tight junctions could be restored by treating these Ras-transformed cells with the MEK1 (mitogen-activated protein kinase kinase) inhibitor PD98059 (Chen *et al.*, 2000). Our results provide another potential linkage between the Ras pathway and junctional complex regulation, via the interaction of AF-6 and ZO-3.

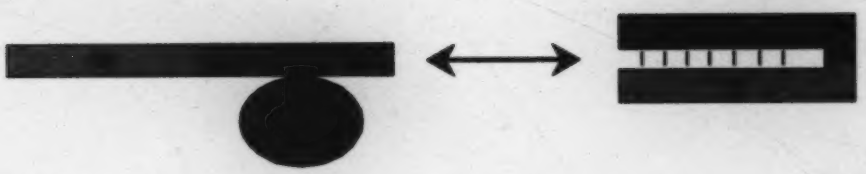
The results presented here also reveal that FLZO-3 and CZO-3 interact with p120 catenin from MDCK lysate. This is the first demonstration of a tight junction protein interacting with p120 catenin, although binding of tight junction proteins to another catenin has been shown before. Both ZO-1 and ZO-2 interact with α-catenin (Itoh *et al.*, 1997; Itoh *et al.*, 1999). The dual role of p120 catenin in cell-cell adhesion and cell migration make it an ideal candidate to link together tight junction elements and the Rho GTPases in the NZO-3-induced phenotype.

*4.4.4. A Possible Mechanism to Link NZO-3 Expression to the Rho Pathway*

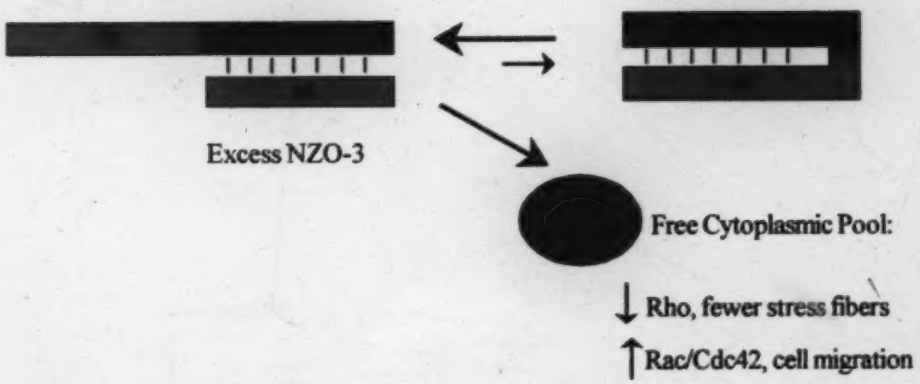
Thinking of a molecular mechanism that would explain how exogenous expression of NZO-3 alters the activity of the Rho GTPase, while it is the COOH-terminal half of ZO-3 that binds p120 catenin and AF-6 is not immediately intuitive. A potential mechanism we want to test involves competition between intramolecular binding interactions and intermolecular binding interactions. This theory is presented in Figure 4.9. It is possible that the N-terminal half of ZO-3 can bind to the COOH-terminal half of ZO-3 of the same molecule (intramolecular interaction) via the COOH-terminal SH3 domain and a consensus PXXP binding motif in the proline-rich region of the N-terminal half of the molecule. In this case, it is possible there is competition for p120 catenin binding to the same region of CZO-3. In parental MDCK cells (Figure 4.9, part A), both interactions could occur. In NZO-3/MDCK cells however, the exogenous NZO-3 present could compete for p120 catenin binding to the COOH-terminus of the endogenous ZO-3 (Figure 4.9, part B). This situation could then cause an increase in the soluble pool of p120 catenin, subsequent activation of Rac/Cdc42 through Vav2, and inhibition of RhoA as observed in previous studies (Noren *et al.*, 2000). The end result of this signaling pathway is a decrease in the amount of stress fibers and increased motility in these experiments (Noren *et al.*, 2000). We have not yet been successful at determining the solubility of p120 catenin in NZO-3/MDCK cells; further work will be necessary to clarify this issue. Whether an interaction between NZO-3 and CZO-3 occurs, and if this binding is mutually exclusive to p120 catenin binding can also be resolved by direct binding assays. Finally, in the case of CZO-3/MDCK cells

**Figure 4.9. Hypothetical model for the mechanism of RhoA inhibition in NZO-3/MDCK cells.** A. In parental MDCK cells, the COOH-terminus of endogenous ZO-3 could bind to p120 catenin, or possibly to the N-terminus of the same ZO-3 molecule (intramolecular interaction). B. In NZO-3/MDCK cells, excess exogenously-expressed NZO-3 could compete with p120 catenin for binding to the COOH-terminus of endogenous ZO-3, thus increasing the soluble pool of p120 catenin. Soluble p120 catenin has been shown to cause a decrease in RhoA activity, and an increase in Rac/Cdc42 activity concomitant with increased motility (Noren *et al.*, 2000). It is thought that soluble p120 catenin interacts with the Rac GEF, Vav2 to mediate this effect. C. In CZO-3/MDCK cells, p120 catenin could bind to either the COOH-terminus of endogenous ZO-3, or the CZO-3 construct, thus sequestering it from binding to Vav2 and causing the downstream effects in the cytoplasm as in part B.

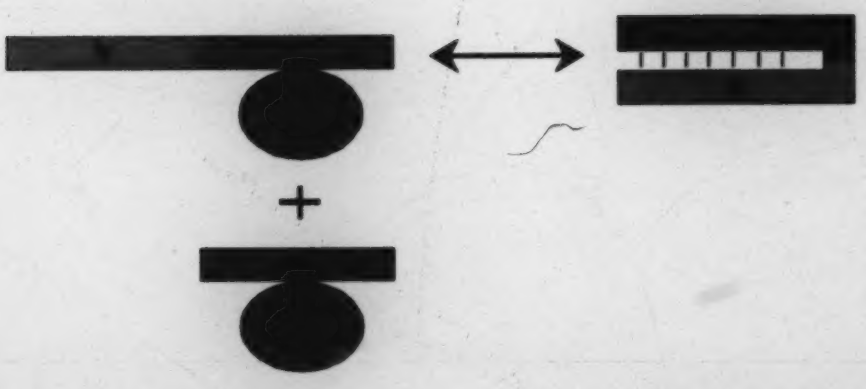
A. Parental MDCK cells



B. NZO-3/MDCK cells




C. CZO-3/MDCK cells



Intermolecular Interactions vs. Intramolecular Interactions

(Figure 4.9, part C), p120 catenin could bind to the COOH-terminus of the endogenous ZO-3 or the exogenous CZO-3 fragment. Binding of p120 catenin to the CZO-3 fragment may sequester p120 catenin and prevent it from interacting with other binding partners (i.e. Vav2) in the cytoplasm. This would also provide an explanation for the increased RhoA activity observed in CZO-3/MDCK cells compared to parental cells (Figure 4.6).

Regulation of protein function via intramolecular interactions is an established paradigm. For example, ezrin (an ERM family protein) exists in two conformationally distinct states: a "dormant" state, which is a closed conformation where the N-terminus of the protein binds to the COOH-terminus in a head-to-tail manner, and an "active" state that opens up the protein and exposes otherwise masked binding sites to allow other intermolecular binding interactions to take place (Bretscher *et al.*, 2000). In the case of ezrin, the active state is able to interact with the membrane via its N-terminus and the cytoskeleton via its COOH-terminus (Bretscher *et al.*, 1997). Another example of a protein that is regulated by intramolecular interactions is vinculin. In the case of vinculin, the loss of the intramolecular interaction of the head domain and tail domain accounts for the increased affinity for talin of the head domain compared with intact vinculin (Johnson and Craig, 1994). Disruption of the head-tail interaction also reveals an otherwise hidden F-actin binding site in the tail region of vinculin (Johnson and Craig, 1995). Finally, another MAGUK protein, SAP97, has been shown to possess this type of regulation; where an N-terminal domain interacts with the SH3 and GUK domain of the same molecule, thus altering its other protein-protein interactions (Wu *et al.*, 2000). While the model in Figure 4.9 is speculative, it offers a potential mechanism to explain

how expression of the amino terminal-half of ZO-3 causes a decrease in RhoA activity levels and altered actin cytoskeleton dynamics in NZO-3/MDCK cells.

*4.4.5. Conclusions*

In summary, the results presented in this chapter indicate a novel link between a tight junction protein and cell signaling pathways influencing actin cytoskeleton regulation. Expression of the N-terminal half of the tight junction protein ZO-3 caused a noticeable decrease in the amount of stress fibers and fewer and smaller focal adhesions which resulted in increased migratory ability of these cells. We correlated this actin phenotype and increased migration with decreased RhoA activity in NZO-3/MDCK cells. Finally, we show that the COOH-terminal half of ZO-3 binds to both AF-6 and p120 catenin, and we hypothesize a model where altered binding interactions involving ZO-3, p120 catenin and possibly AF-6 in NZO-3-expressing cells induce Rho GTPase signaling, leading to the actin cytoskeleton/migration phenotype. The results presented here indicate possible linkages between tight junction proteins and Rho GTPase-related signaling events.

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# CHAPTER 5

## General Discussion

## 5.1. Overview

The field of tight junction biology has undergone an explosion of knowledge in recent years since the identification of the first tight junction protein, ZO-1, just over 15 years ago (Stevenson *et al.*, 1986). Relative to other cell-cell interaction fields such as the gap junction field which identified its first protein component, connexin, 12 years earlier (Goodenough, 1974), the tight junction field is just emerging from its infancy. The groundwork has been laid through the identification of a great number of tight junction-associated proteins (Table 1.1), and now the challenge is to elucidate how these proteins function, interact, and assemble to form a tight junction. Much remains unknown regarding specific tight junction protein function, and how the molecular organization of these proteins establishes a regulated paracellular permeability barrier.

The main focus of our laboratory in recent years has been the identification and characterization of the tight junction protein ZO-3 (Haskins *et al.*, 1998). ZO-3, together with two other previously identified tight junction proteins ZO-1, and ZO-2 (Stevenson *et al.*, 1986; Jesaitis *et al.*, 1994; Beatch *et al.*, 1996), is a member of the MAGUK protein family that functions in the organization of molecular complexes at the plasma membrane. The goal of this thesis project was to further characterize the molecular interactions among tight junction proteins, and begin to discern the function of the tight junction protein ZO-3. For reference, Figure 5.1 is a schematic representation of the key molecular interactions among tight junction proteins, and specifically highlights the binding interactions that were identified through the work presented in this thesis.

The results presented in Chapter 2 provide information regarding the binding interactions involving ZO-1, ZO-2, ZO-3, and occludin, and also explore the molecular

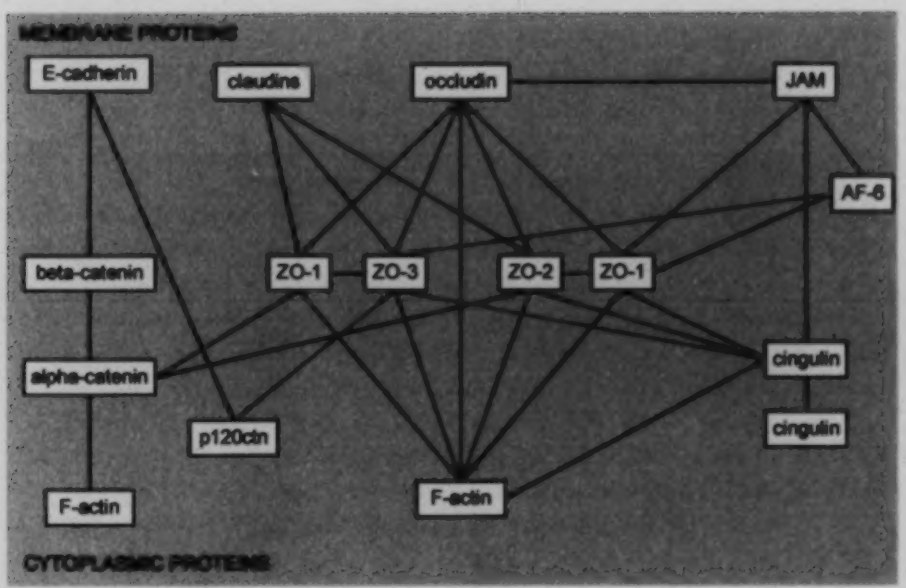


**Figure 5.1. Schematic representation of key molecular interactions involving junctional complex proteins.** Note the complexity and apparent redundancy in binding interactions among tight junction proteins and F-actin in contrast to the adherens junction. Binding partners joined with a red line indicate results obtained in this thesis project. Whether all of these interactions occur simultaneously, or if preferential sub-groupings occur *in vivo* remains to be determined. See text for references.

mechanism for F-actin association with the tight junction plaque. In Chapter 3, I focused on the further characterization of ZO-3; its binding interactions, and its role in junctional complex assembly, with the ultimate goal of relating information about protein function to tight junction physiology. The results from Chapter 3 lead us in an unexpected direction, with the discovery that expression of truncated ZO-3 induced changes in actin cytoskeleton morphology. We explored this phenomenon further in Chapter 4, where we identify a putative link between the tight junction protein ZO-3 and the RhoA family of GTPases that play key roles in actin cytoskeleton dynamics. The following sections will summarize the data obtained in each chapter, discuss problems that remain to be solved, and future experiments that will address these issues.

## 5.2. Chapter 2: Binding Interactions

### *5.2.1 Linkage to the Actin Cytoskeleton*

How the tight junction proteins ZO-1, ZO-2, and ZO-3 interact with each other and other tight junction proteins is of obvious interest considering their proposed scaffolding role as MAGUK protein family members, and in this chapter I provide information on a number of binding interactions. Actin cosedimentation studies showed that ZO-2, ZO-3, and occludin all interact directly with F-actin in vitro, ZO-2 binds directly to both ZO-1 and occludin, and contrary to previous beliefs, ZO-1, ZO-2, and ZO-3 exist *in situ* primarily as independent ZO-1/ZO-2 and ZO-1/ZO-3 complexes rather than a trimeric ZO-1/ZO-2/ZO-3 grouping. It is important to reiterate that all the binding experiments performed in this chapter used purified recombinant proteins, so that we can confirm that all the interactions are direct. I believe direct binding data allow greater

reliability when predicting molecular mechanisms related to tight junction biology. In contrast, other investigators have presented experimental results suggesting that ZO-1 and ZO-2 bind each other and that ZO-1 and ZO-2 bind to occludin and F-actin (Furuse *et al.*, 1994; Itoh *et al.*, 1997; Fanning *et al.*, 1998; Itoh *et al.*, 1998), but these experiments were performed using whole cell lysates, raising the possibility that unidentified proteins present in the lysate are mediating the observed interaction.

In Chapter 2, we have confirmed that ZO-2 binds directly to ZO-1 and occludin. We also confirmed that ZO-2 binds directly to F-actin and extend this result by showing that ZO-3 and occludin also bind directly to F-actin. It is interesting to note that the ZO-1/F-actin interaction has not to date been proven to be direct (i.e., through the use of purified proteins), although given the sequence similarity to ZO-2 and ZO-3, this likely is the case. The fact that F-actin has so many possible linkage points to transmembrane tight junction proteins is in contrast to the adherens junction, where the main linkage to E-cadherin is through α-catenin (refer to Figure 5.1). The tight junction is also unique in that it doesn't exclusively follow the paradigm of transmembrane proteins being connected to the actin cytoskeleton via cytoplasmic linker proteins, since occludin can bypass this and bind to F-actin directly. It is also important to note that whether all of these possible binding interactions exist at the tight junction simultaneously, or if there are preferential interactions depending on the physiological state of the tight junction remains to be determined.

*5.2.2. ZO-1, ZO-2, and ZO-3 Binding Interactions*

Prior to these studies, the dogma in the field was that ZO-1, ZO-2, and ZO-3 exist in a three-membered protein complex at the tight junction. This was based on the

observation that ZO-1 antibodies coimmunoprecipitate ZO-2 and ZO-3 from MDCK cell lysate (Gumbiner *et al.*, 1991; Balda *et al.*, 1993; Jesaitis *et al.*, 1994; Haskins *et al.*, 1998). In fact, both ZO-2 and ZO-3 were originally identified on this basis. We challenged this notion of a ZO-1/ZO2/ZO-3 trimeric complex by performing coimmunoprecipitations using antibodies to ZO-1, ZO-2, and previously unavailable ZO-3 antibodies. Our results indicate that independent ZO-1/ZO-2 and ZO-1/ZO-3 heterodimeric complexes exist. If a trimeric ZO-1/ZO-2/ZO-3 complex exists, it is either extremely rare or it could be a transient phenomenon that escapes detection with our methods. The observation that the balance between the two complexes is shifted in favor of ZO-1/ZO-3 in cells that express exogenous ZO-3 indicates that ZO-3 can compete with ZO-2 for binding to ZO-1 if ZO-3 levels are not limiting. Whether ZO-3 has a higher affinity for ZO-1 than ZO-2 remains to be determined. It is known that ZO-1 and ZO-2 interact via their PDZ2 domains (Fanning *et al.*, 1998; Itoh *et al.*, 1999), and it is likely that ZO-3 binds to ZO-1 in the same way. We have not determined the exact ZO-1 binding site on ZO-3, except that it is somewhere in the PDZ domain-containing N-terminus of ZO-3 (see Figure 3.11), which is consistent with the hypothesis that it interacts via the PDZ2 domain. More research needs to be done to narrow down the exact binding regions of interacting tight junction proteins. The binding results in this thesis along with work from other labs will help provide a framework on which to base these future experiments.

## 5.3. Chapter 3: The Role of ZO-3 During Junctional Complex Assembly

In this chapter, the functional characteristics of the tight junction protein ZO-3 were explored by exogenously expressing partial constructs in MDCK cells and looking at the mutant phenotype(s) generated. Expression of the N-terminal, PDZ domain-containing half of the molecule (NZO-3) delayed the assembly of tight junctions as measured by TER levels, and this correlated with delayed recruitment of ZO-1, ZO-2, endogenous ZO-3, occludin, F-actin, the adherens junction proteins E-cadherin and β-catenin to the junctional membrane. NZO-3 expression did not alter the amount of ZO-1, ZO-2, endogenous ZO-3, occludin, or E-cadherin present in the cell; however, the amount of Triton X-100-soluble, signaling-active β-catenin was increased. Finally, *in vitro* binding experiments showed that ZO-1 and actin preferentially bind to NZO-3, whereas both NZO-3 and the carboxy-terminal half of the molecule (CZO-3) contain binding sites for occludin and cingulin.

### *5.3.1 Junctional Assembly*

In our experiments we used TER recovery and recruitment of junctional proteins to the cell membrane after calcium switch as measures of junctional assembly. The spike in TER in all cell lines except the NZO-3/MDCK cell line is a phenomenon that has been consistently observed by many independent investigators in this field, but has never been fully accounted for. Recently, Furuse and colleagues (Furuse *et al.*, 2001) showed that individual claudin family members impart varying levels of TER on tight junctions. For example, they found that claudin-2 is specific for the more permeable MDCK type II strain, but is absent in the higher resistance strain MDCK type I. By transfecting claudin-2 into the type I strain they were able to revert it to a more permeable phenotype, with a

similar TER to MDCK II cells. Due to this observation, that introduction of one specific claudin can completely change the permeability characteristics of the cells, they speculate that variation of the tightness of tight junction strands is determined by the combination and mixing ratios of claudin species. In further support of this, another claudin, paracellin-1 (claudin 16), can actually form pores through homophilic interactions between adjacent cells, and it is through these pores that $Mg^{2+}$ ions are selectively reabsorbed (Simon *et al.*, 1999). Perhaps the spike in TER early in the junctional complex assembly process is related to recruitment of claudin family members with different permeability characteristics at different times during junctional assembly.

When we looked at recruitment of junctional proteins during assembly, we observed that not only does NZO-3 expression affect tight junction protein localization, but F-actin and the adherens junction proteins E-cadherin and β-catenin also show delayed recruitment to the cell membrane. This result supports the theory that tight junction and adherens junction assembly is a coordinately regulated process, and that NZO-3 expression negatively affects one or more steps in this process. While it has been shown that disruption of an adherens junction component (i.e., E-cadherin) prevents tight junction protein recruitment to the membrane during junctional complex assembly (Gumbiner and Simons, 1986; Gumbiner *et al.*, 1988), we now present the novel result that the converse is also true.

*5.3.2. Protein Levels*

We hypothesized that NZO-3 might exert its effects by lowering or increasing the amounts of other junctional proteins within the cell. This scenario could occur if the presence of NZO-3 in the cell alters the stability of other proteins during junctional

assembly. NZO-3 expression did not alter amount of ZO-1, ZO-2, endogenous ZO-3, occludin, or E-cadherin; however, the amount of Triton X-100-soluble, signaling-active β-catenin was increased (see below). A very intriguing result was that there was an increase in NZO-3 protein levels at early time points of the calcium switch (6 h), before declining and returning to steady-state levels by 48 h. The peak of NZO-3 protein levels corresponds to the time period where TER recovery is lagging and junctional components are still mislocalized; once NZO-3 levels decrease, steady-state TER is attained and junctional proteins are correctly localized. Therefore, there is a temporal correlation of the times of high NZO-3 levels to the times of greatest junctional disruption. The NZO-3 expression construct is under control of a constitutive promoter (CMV), therefore one would expect the protein levels to be constant. The variance of NZO-3 protein level over the time course of junctional assembly could be related to differences in stability of the construct; it is possible that at early time points it exhibits a different set of binding partners that stabilize it. Pulse-chase analysis during a calcium switch experiment might prove fruitful in determining the stability of NZO-3 over the time course of junctional assembly. Recently, work from our laboratory has shown that this spike in protein levels at early time points is a feature specific to the NZO-3 construct. A CZO-3/MDCK cell line with the CZO-3 construct expressed under the same promoter does not exhibit this peak, and protein levels remain constant throughout the time course of the calcium switch (J. Haskins, unpublished data). It is possible that stabilizing protein interactions take place at early but not late stages of junctional assembly. Alternatively, the NZO-3 mRNA might be unstable and targeted for degradation before being translated. One could test this hypothesis by performing northern blot analysis of NZO-3 mRNA levels

during the course of junctional assembly. It is also possible that the cell has alternate mechanisms of junctional assembly that occur at latter stages, which are able to overcome the inhibitory effect of NZO-3-expression.

*5.3.3. Possible Involvement of the β-catenin Signaling Pathway*

Not only did expression of NZO-3 cause β-catenin to be mislocalized at early stages of junctional assembly, but there was also an increase in the TX-100-soluble pool of signaling-active β-catenin. Soluble cytoplasmic β-catenin is capable of translocating to the nucleus where it acts as a transcriptional transactivator in a complex with TCF/LEF family of transcription factors to direct transcription of a variety of genes (e.g. cyclin D1) that promote a proliferative phenotype (Behrens *et al.*, 1996; Huber *et al.*, 1996; Tetsu and McCormick, 1999). It is possible that the presence of high levels of NZO-3 at early time points of junctional assembly results in release of β-catenin from the cadherin complex, and this is what causes the increased TX-100-soluble pool. It remains to be tested whether ZO-3 binds to E-cadherin, but a possible mechanism for increased soluble β-catenin is competition for binding to E-cadherin. ZO-3 and β-catenin do not coimmunoprecipitate from MDCK cells (data not shown). Given the results in Chapter 4 regarding the involvement of p120 catenin in NZO-3/MDCK cell phenotype, we cannot discount the possibility that the observed phenotype is due to p120 catenin entering the nucleus and interacting with certain transcription factors (Daniel and Reynolds, 1999).

*5.3.4. Protein Interactions*

Finally, *in vitro* binding experiments showed that ZO-1 and actin preferentially bind to NZO-3, whereas both NZO-3 and the carboxy-terminal half of the molecule (CZO-3) contain binding sites for occludin and cingulin. It will be interesting to

determine if there are two distinct binding sites for occludin and cingulin on ZO-3, one in each half of the molecule, or if the binding region overlaps the break point. If two distinct binding sites are present on the ZO-3 molecule, it will be important to ascertain whether there is a preference for one particular site, or if one cingulin or occludin molecule can bind two ZO-3 molecules simultaneously. More specific domain binding analyses with purified recombinant proteins is one means to answer this question.

NZO-3 specifically binds ZO-1 and F-actin, while CZO-3 does not. F-actin has multiple possible binding partners at the tight junction in addition to ZO-3 (refer to Figure 5.1), and it can be envisioned that this arrangement provides the means by which actin filaments are securely associated with the tight junction in order to apply the tension needed for barrier regulation. Because the N-terminal half of ZO-3 is responsible for binding F-actin, this may represent one mechanism whereby expression of this construct affects junctional complex assembly, although the apparent redundancy of having multiple other binding partners for F-actin at the tight junction may compensate.

### 5.4. Chapter 4: NZO-3 Expression has Global Effects on the Actin Cytoskeleton

The perturbations of the actin cytoskeleton in NZO-3/MDCK cells identified in Chapter 3 prompted us to look at other aspects of actin cytoskeleton regulation and actin-dependent events within this cell line. NZO-3/MDCK cells have fewer actin stress fibers and focal adhesions concomitant with faster cell migration relative to untransfected MDCK cells or CZO-3-expressing cells, and this can be correlated with decreased Rho GTPase activity. We have begun to explore the mechanism responsible for this decrease

in RhoA activity, and our results suggest a link to p120 catenin, a protein noted for having dual roles in cell migration versus cell adhesion (Noren *et al.*, 2000).

*5.4.1 NZO-3 Expression Induces Cell Migration*

We used a monolayer wound healing assay as a measure of the migratory ability of our cell lines. Another type of motility assay, the Transwell migration assay, measures the ability of cells to separately migrate from one side of porous membrane support to the other. This type of migration is typically observed in fibroblasts, or cells that have lost their cell-cell contacts, for example during tumor metastasis. We did not perform Transwell migration assays because our cells stay in contact with each other and migrate as a cell sheet during the wound healing assay, therefore, we feel that our experiments sufficiently demonstrate the increased migratory ability of NZO-3/MDCK cells.

One puzzling aspect about the observed increased rate of migration of NZO-3/MDCK cells is that the cells at the free-edge of the wound do not show obvious increases in lamellipodial or filopodial structures, and in fact have less intense F-actin staining compared to parental MDCK cells at the leading edge (Chapter 4). One explanation for this might be that the F-actin at the leading edge of NZO-3/MDCK cells is more dynamic, with a more rapid turnover that propels the cell forward. To test this, it would be interesting to stain wounded monolayers for Arp2/3, a protein complex that plays a major role in nucleating *de novo* actin polymerization and can organize actin networks at the cell periphery, promoting protrusion and migration (Higgs and Pollard, 1999).

*5.4.2. Role of the Rho family of GTPases*

The decrease in RhoA activity we observed in NZO-3/MDCK cells is exciting as a potential explanation for the stress fiber phenotype and increased cell migration of these cells, but it may not be the whole story. Rac and Cdc42 activation can also induce cell migration directly, through the formation of filopodia and lamellipodia (Ridley *et al.*, 1992; Nobes and Hall, 1995) or indirectly via inhibition of RhoA (Sander *et al.*, 1999). Rac and Cdc42 activity assays are currently in progress to answer this question. It is quite possible that it will be a combination of RhoA inactivation to decrease the attachment of the cell to the substratum, and Rac/Cdc42 activation to induce the membrane protrusive structures at the leading edge that causes the increased migration rate in NZO-3/MDCK cells. To add another layer of complexity, the activation state of these GTPases might be spatiotemporally regulated within the cell cytoplasm. An example of this is the localized RhoA inhibition by p190RhoGAP at the leading edge of migrating cells (Arthur and Burridge, 2001).

*5.4.3. Binding Interactions: ZO-3, p120 catenin, and AF-6*

Our search for binding partners of ZO-3 was a way to gain information about the molecular mechanism underlying the phenotypes we observe in NZO-3/MDCK cells. We did not find an interaction between NZO-3 and RhoA, Rac, or Cdc42 (Figure 4.7), although we have not yet tested whether CZO-3 can bind these proteins. It is also possible that ZO-3 interacts with a GTP dissociation inhibitor (GDI) or a guanine nucleotide exchange factor (GEF), and this remains to be tested. However, we identified that AF-6 and p120 catenin both bind preferentially to the COOH-terminal half of ZO-3 (Figure 4.8). We have proposed a model for how interactions involving ZO-3 might

cause the downstream RhoA inhibition in NZO-3/MDCK cells (Figure 4.9). This is a working model based on the data we have currently. One part of the model that needs to be tested is whether the solubility of p120 catenin is increased in NZO-3/MDCK cells compared to control cells. We will also need to perform direct *in vitro* binding experiments to determine if the NZO-3 construct interacts with CZO-3. Finding the answers to these questions will help validate the working model outlined in Figure 4.9. The data presented in this chapter identify a possible link between the tight junction protein ZO-3 and the Rho family of GTPases, however it is clear that there are many questions remaining to be answered.

### 5.5. Overall Conclusions

Since the discovery of the first tight junction protein, ZO-1 (Stevenson *et al.*, 1986), the field of tight junction biology has grown at a rapid pace; however, key questions remain. One challenge is to determine the complete catalogue of tight junction proteins that reflects the dynamic nature of the tight junction. Progress is being made in this quest, as there are now close to thirty proteins that have been localized to the tight junction (Table 1.1). The search for novel proteins in the future may be aided by the use of a proteomics approach using partially purified subcellular fractions containing tight junctions. As new molecular components are discovered, the next challenge will be to elucidate the function of each protein. For example, how are these proteins assembled at the membrane into a functional complex? What are the binding interactions between the different tight junction proteins, and can alterations in these interactions produce different permeability characteristics? One particular area in this field that is lacking functional

information is that of signal transduction: what are the exact signaling pathways controlling tight junction assembly/disassembly and physiological regulation of paracellular permeability? Finally, in a broader context, what role does the tight junction play during disease processes? Disruption of the tight junction is a hallmark of many diseases including Crohn's disease, inflammatory bowel disease, diabetic retinopathy, and the pathophysiology of food poisoning caused by *Clostridium perfringens* (Madara, 1998; Sonoda *et al.*, 1999). Although progress is being made in understanding tight junction biology, much more work remains to be done. The results presented in this thesis provide a basis for some of this future work.

**5.6. Bibliography**

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